Mechanisms of Fecal Microbiota Transplantation and Development of Novel Therapies for *Clostridium difficile* Infection

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Abstract

Clostridium difficile infection is the most common nosocomial infection in the US and other developed countries, yet standard antibiotic therapy for this infection fails to cure nearly a quarter of patients. These individuals experience a recurrence of the infection, and a large subset of patients with a single recurrence go on to develop recurrent *C. difficile* infection syndrome (R-CDI), characterized by vicious cycles of repeated antibiotic use and recurrence of disease. Although fecal microbiota transplantation (FMT) is now widely recognized as an extremely effective therapy for R-CDI, its mechanisms were heretofore unknown. This substantial gap in our knowledge limited both our ability to design novel therapies for R-CDI and our understanding of the role our native intestinal microbes play in our health.

The work herein summarizes our efforts to identify and test the mechanisms behind this important treatment. Our findings indicate that fecal bile acid composition, which is significantly altered following FMT, also dramatically impacts *C. difficile* physiology. Bile acids present after FMT, compared to those present prior to the procedure, prevent germination of *C. difficile* spores and limit the growth of vegetative cells. With this knowledge, we demonstrated that a bile acid already clinically available, ursodeoxycholic acid (UDCA), can also prevent *C. difficile* germination and growth. Although unfortunately UDCA is a poor treatment option for many patients due to its absorption in the small intestine, we showed both that a non-absorbed derivative of UDCA, C7-sulfated UDCA, may be a promising novel therapy for R-CDI, and that UDCA may still have clinical utility in patients with altered gastrointestinal anatomy. These findings have paved the way for novel treatments for this widespread infection.

Supplementary Files

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Chapter 1

Introduction

Clostridium difficile infection and basic biology

Each year in the US, nearly half a million individuals suffer from hospital-acquired infections with the bacterium *Clostridium difficile* (Lessa et al., 2015). This bacterium enters the gastrointestinal tract through the fecal-oral route and, typically in patients who have recently received antibiotics (Kelly and Lamont, 2008; Miller et al., 2011; Ananthrakishnan, 2011; Lessa et al., 2012; Khanna et al., 2012; Chitnis et al., 2013; Borody and Khoruts, 2011; Hu et al., 2009; Fashner et al., 2011), colonizes the colon and causes inflammation, abdominal pain, and diarrhea (Cohen et al., 2010; Kachrimanidou and Malisiovas, 2011). Some unlucky patients may experience more severe clinical sequelae, including toxic megacolon, ileus, and pseudomembranous colitis (Cohen et al., 2010).

Unfortunately, in addition to causing debilitating symptoms, *Clostridium difficile* infection (CDI) is extremely common. CDI has surpassed methicillin-resistant *Staphylococcus aureus* as the most common nosocomial infection in the US (Zilberberg et al., 2008; Miller et al., 2011; Dubberke and Olsen, 2012; Magill et al., 2014). Furthermore, it is increasingly recognized that CDI can occur in a community (rather than hospital) setting; between 32% and 41% of CDI cases are predicted to be community-associated (Khanna et al., 2012; Lessa et al., 2014; Lessa et al., 2015). Unsurprisingly the costs associated with this common infection are staggering; in 2008 alone it is estimated that excess healthcare costs associated with treating CDI may have been as high as $4.8 billion (Dubberke and Olsen, 2012). Furthermore, CDI was responsible for the deaths of 2600 individuals in the US in 2000, which rose to over 14,000 in 2007 (Hall et al., 2012).

The most recent epidemiological data, from 2011, estimates over 29,000 deaths are due to CDI each year (Lessa et al., 2015). The healthcare and economic burden of CDI, complicated by its increasing resistance to standard antibiotic therapies (McDonald et al., 2005; Vardakas et al., 2012), make this an important disease to understand and treat effectively.

The causative agent of CDI, the bacterium *Clostridium difficile*, is a Gram-positive, anaerobic, spore-forming rod which possesses a Pathogenicity Locus (PaLoc) encoding the toxins responsible for disease symptoms. These toxins, Toxin A and Toxin B (TcdA and TcdB), are Rho-glucosylating exotoxins which bind to human epithelial cells and cause inflammation, edema, neutrophil recruitment, and cytoskeleton depolymerization (Linevsky et al., 1997; Voth and Ballard, 2005; Nam et al., 2010; Kachrimanidou and Malisiovas, 2011). The relative importance of each toxin in CDI pathogenesis is not entirely clear, though evidence suggests that TcdB, but not TcdA, is essential for virulence (Lyras et al., 2009). The PaLoc also encodes a sigma factor (TcdR) which promotes transcription of *tcdA* and *tcdB*, a putative holin (TcdE), and a negative transcriptional regulator (TcdC) (Kachrimanidou and Malisiovas, 2011).

Mutations in some of these PaLoc genes as well as other mechanisms may account for the emergence of hypervirulent strains of *C. difficile*, particularly the North American pulsed-field gel electrophoresis type 1 (NAP1) strain, which is also described as restriction endonuclease analysis group BI and polymerase chain reaction ribotype 027 (McDonald et al., 2005; Loo et al., 2005; Warny et al., 2005). NAP1/BI/027 isolates contain mutations in *tcdC*, which may increase toxin production (McDonald et al., 2005). These isolates also produce another toxin, the binary toxin or CDT, which is encoded at a

separate locus from TcdA and TcdB and also causes cytoskeleton disruption (Kachrimanidou and Malisiovas, 2011; Perelle et al., 1997). In addition to increased or alternative toxin production, hypervirulent strains of *C. difficile* are also resistant to fluoroquinolone antibiotics (McDonald et al., 2005) and sporulate with greater efficiency than other strains (Merrigan et al., 2010). Together, these characteristics may help explain why NAP1/BI/027 isolates are frequently responsible for major outbreaks of CDI (McDonald et al., 2005) as well as clinically severe and recurrent cases of the infection (Marsh et al., 2012; See et al., 2014).

Sporulation and germination of *C. difficile*

In particular, the sporulation of NAP1/BI/027 isolates and other *C. difficile* strains is thought to be critical for CDI pathogenesis. *C. difficile* spores are highly resistant to disinfectants and antibiotics (Lawley et al., 2010; Carroll and Bartlett, 2011; Paredes-Sabja et al., 2014) and can survive on hospital surfaces for at least five months (Kim et al., 1981). As with other spore-forming bacteria such as *Bacillus subtilis*, the transcriptional regulator Spo0A appears to be vital for sporulation in *C. difficile* (Underwood et al., 2009) and is also required for persistence and transmission of the pathogen (Deakin et al., 2012). However, both the upstream regulation and activation of Spo0A and its downstream targets are less well understood in *C. difficile* than in other organisms. In other species, several orphan histidine kinases phosphorylate Spo0A to initiation sporulation (Steiner et al., 2011; Higgins and Dworkin, 2012). While several orphan histidine kinase genes have been discovered in the *C. difficile* genome (Underwood et al., 2009), it is currently unclear whether these histidine kinases participate in sporulation. Following phosphorylation, Spo0A initiates a cascade of sporulation-specific sigma factors which ultimately direct the process of sporulation (de Hoon et al., 2010). In *C. difficile*, four sigma factors have been identified in this cascade, including two (σ^E and σ^K) in the mother cell and two (σ^F and σ^G) in the forespore (Paredes-Sabja et al., 2014). In contrast to *B. subtilis*, however, there is substantially less, if any, cross-talk between the mother cell and forespore using these sigma factors (Fimlaid et al., 2013; Pereira et al., 2013; Saujet et al., 2013). Despite these recent

insights into *C. difficile* sporulation, the noticeable differences in sporulation mechanisms between *C. difficile* and other organisms suggest there is much left to understand.

Even the relative paucity of knowledge concerning *C. difficile* sporulation, however, is much greater than what is currently known about the germination of the resulting spores. In *Bacillus subtilis*, germinants such as amino acids, sugars, or nucleotides bind to the germinant receptor, triggering the release of Ca^{2+}-dipicolinic acid (DPA) from the specialized spore coat, followed by hydrolysis of the cell cortex, loss of resistance, loss of dormancy, and ultimately outgrowth (Setlow, 2003). However, recent work has demonstrated that in *C. difficile*, cortex hydrolysis precedes and may even trigger DPA release, suggesting that germination in *C. difficile* proceeds via a novel pathway (Francis et al., 2015).

In support of the idea that the process of germination in *C. difficile* is distinct from other bacteria, very little is known about the underlying mechanisms. Recent bioinformatics analyses have unfortunately uncovered very few genes which are homologous to other known germinant receptors and spore proteins (Sebaihia et al., 2006; Sebaihia et al., 2007; Lawley et al., 2009). However, it has been recently demonstrated that CspC, an analog of a subtilisin-family of proteases in which have been shown to activate the cortex lytic enzyme SleC to induce germination in *Clostridium perfringens*, may be a germinant receptor for *C. difficile* (Francis et al., 2013). In *C. difficile*, CspC has lost its catalytic domain, but is necessary for germination and required for infection in a hamster model of CDI (Francis et al., 2013). Beyond this work, however, little is known about how *C. difficile* senses germinants and initiates the process of germination.

Despite minimal knowledge of the genetics and regulation of *C. difficile* germination, there is a growing body of work focused on the specific germinants which trigger the process. Most of these germinants are bile acids, molecules produced by the human liver to aid lipid digestion in the small intestine (Hofmann, 1999). The bile acids directly produced and secreted by the liver as known as primary bile acids (Hofmann, 1999). Although ~95% of primary bile acids are absorbed by the small intestine and returned to the liver via enterohepatic circulation (Hofmann and Hagey, 2008), around 5% enter the colon, where they are modified by native intestinal bacteria to form the secondary bile acids (Ridlon et al., 2006). The two major primary bile acids in humans are cholate (CA) and chenodeoxycholic acid (CDCA) (Hofmann and Hagey, 2008). During production in the liver, the majority of these primary bile acids are conjugated to either glycine or taurine to increase solubility, then stored in the gallbladder until they are released post-prandially to assist in digestion (Russell and Setchell, 1992; Hofmann, 1999; Hoffman and Hagey, 2008).

Upon reaching the colon, the first transformation these bile acids undergo is the removal of the conjugated taurine or glycine by bacterial bile salt hydrolases (BSHs) (Ridlon et al., 2006). BSHs are widespread among colonic microbes, as these genes have been identified in the *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* phyla, as well as in the human gut archaeon *Methanobrevibacter smithii* (Jones et al., 2008). After deconjugation, CA and CDCA can be metabolized into their respective secondary bile acids, deoxycholate (DCA) and lithocholic acid (LCA), which comprise most of the bile acids found in feces (Ridlon et al., 2006). This process is known as 7α-dehydroxylation, and in contrast to deconjugation, is performed by a very limited number of intestinal

organisms. It is currently estimated that only approximately 0.0001% of colonic bacteria can perform 7α-dehydroxylation, all of which are exclusively members of the *Clostridium* genus (Wells et al., 2000; Wells et al., 2003; Ridlon et al., 2006). The genes responsible for 7α-dehydroxylation are encoded by the bile acid inducible (*bai*) operon, which has been well-characterized in two species, *Clostridium scindens* and *Clostridium hylemonae*, both of which are members of the *Lachnospiraceae* family (Ridlon et al., 2010).

All of these bile acids, primary and secondary, appear to have important effects on *C. difficile* physiology. It has been recognized for decades that the addition of taurocholate (TA), the taurine conjugate of CA, to laboratory media is essential for *C. difficile* growth (Wilson et al., 1982; Buggy et al., 1985). Specifically, TA, as well as CA, has been shown to cause germination of *C. difficile* (Wilson, 1983; Sorg and Sonenshein, 2008). It has also been suggested that DCA, the secondary bile acid derived from CA, can induce germination (Sorg and Sonenshein, 2008). In contrast, the primary bile acid CDCA and its secondary bile acid derivative LCA seem to inhibit TA-mediated germination (Sorg and Sonenshein, 2009; Sorg and Sonenshein, 2010). Recently, however, it has been shown that different isolates of *C. difficile* may respond different to CDCA: while for the majority of isolates CDCA inhibits germination, on some it has no effect, and in others CDCA may itself induce germination (Heeg et al., 2012).

In addition to affecting germination, many of these bile acids have important effects on vegetative growth of *C. difficile*. While TA appears to have no impact on growth, growth is significantly inhibited by CDCA and DCA (Sorg and Sonenshein, 2008). Furthermore, colony formation from spores, a function of both germination and

vegetative growth, is induced by CA, TA, and glycocholate, but not by CDCA (Wilson, 1983; Sorg and Sonenshein, 2008). In addition to understanding how these bile acids impact growth, and as our knowledge of the mechanisms of germination in *C. difficile* increases, it will important to fully assess the effects of the bile acids in the human colon on *C. difficile* physiology.

Treatment of *C. difficile* infection

An understanding of the physiology of *C. difficile* is vital for effectively treating CDI and investigating new, more effective therapies for the infection. Although antibiotic use is a major risk factor for CDI (Kelly and Lamont, 2008; Borody and Khoruts, 2011; Louie et al., 2011; Surawicz and Alexander, 2011; Fashner et al., 2011), the current recommendation for the treatment of this disease is the use of further antibiotics. Oral metronidazole is the first-line therapy for an initial (mild or moderate) episode, while oral vancomycin with or without intravenous metronidazole is recommended for severe or complicated initial episodes of the disease (Cohen et al., 2010). Recurrences of the disease are also treated with these two antibiotics, and for patients with multiple recurrences a tapered and/or pulsed regimen of vancomycin may be used (Cohen et al., 2010).

While vancomycin and metronidazole remain the primary choices for initial infections, the use of these antibiotics frequently fails to clear the infection, leading to a recurrence of disease once antibiotics are halted. Infection recurs following vancomycin treatment in 24% of patients, while the recurrence rate of metronidazole is 27% (Vardakas et al., 2012). Overall, it is estimated that there are over 83,000 first recurrences of CDI each year (Lessa et al., 2015). Unfortunately, the rate of recurrence for patients who have already experienced a recurrence can be as great as 65% or higher (Khoruts and Sadowsky, 2011), suggesting that patients with recurrent CDI can become refractory to antibiotic therapy. Indeed, some patients enter vicious cycles characterized by repeated

antibiotic use and CDI recurrence, a condition referred to as recurrent CDI syndrome (R-CDI) (Borody and Khoruts, 2011).

With the observed increase in CDI over the past fifteen years, along with concomitant increases in R-CDI (Pepin et al., 2005; Kelly and Lamont, 2008), there has been a push to develop novel therapies for this disease. One recently FDA-approved new treatment for CDI is fidaxomicin, a macrocyclic antibiotic. In a recent Phase 3 clinical trial, fidaxomicin was shown to be equally effective to vancomycin, but was associated with a significantly lower rate of recurrence (Louie et al., 2011). Further work has indicated that, compared to vancomycin, fidaxomicin has a less dramatic impact on the fecal microbiota, sparing the *Bacteroides*/*Prevotella* groups and *Clostridium* clusters IV and XIVa, which may explain the decreased rate of recurrence with the new antibiotic (Louie et al., 2012).

An older but increasingly popular treatment for CDI, particularly those individuals with R-CDI who are refractory to antibiotic therapy, is fecal microbiota transplantation (FMT). First described as an effective treatment for pseudomembranous colitis in 1958 by Eiseman and colleagues (Eiseman et al., 1958), FMT involves the delivery of fecal material from a healthy donor into the gastrointestinal tract of a patient, either by enema (Eiseman et al., 1958; Schwan et al., 1983; Tvede and Rask-Madsen, 1989), nasogastric tube (Aas et al., 2003; MacConnachie et al., 2009; van Nood et al., 2013), or colonoscopy (Lund-Tonnesen et al., 1998; Yoon and Brandt, 2010; Hamilton et al., 2012). The overall rate of clinical resolution following an FMT is ~90%, substantially higher than vancomycin, metronidazole, and even fidaxomicin (Kassam et al., 2013). FMT via lower gastrointestinal delivery (enema and colonoscopy) is especially

efficacious at 91%, compared to upper gastrointestinal delivery (nasogastric tube) at 82% (Kassam et al., 2013). Furthermore, a recent randomized controlled trial comparing FMT to vancomycin determined that FMT was significantly more effective than the antibiotic (van Nood et al., 2013); in fact the study was halted early by the data and safety monitoring board because of the high rate of relapse in control patients. FMT, therefore, is an extremely promising therapy for individuals with R-CDI.

A search for mechanisms of fecal microbiota transplantation and novel therapies

Despite the promise of FMT, many issues with standardization of the procedure, as well as accessibility of the procedure to both patients and physicians, still exist. Many protocols for FMT involve the use of patient-identified donors (frequently spouses or family members) (e.g. Aas et al., 2003; Yoon and Brandt, 2010; Shahinas et al., 2012). While these donations can be successful, recent work indicates both that patients prefer to use an anonymous, standardized donor and that recovery rates are substantially higher for patients who receive material from a standardized donor (Hamilton et al., 2012). Additionally, the protocols for the procedure itself are often poorly-defined, with some protocols calling for "6 to 8 tablespoons" of fecal material (Kelly et al., 2012) or suggesting filtration with paper coffee filters (Aas et al., 2003). Again, recent efforts have been made to standardize and clarify the process as well as define the number of organisms delivered in each transplant (Hamilton et al., 2012). Finally, the emergence of frozen (Hamilton et al., 2012), freeze-dried (Khoruts et al., 2015), and encapsulated (Stollman et al., 2015; Youngster et al., 2015; Hirsch et al., 2015) preparations should allow the procedure to be done without waiting for a fresh sample from a donor, increasing the ease with which FMTs are performed. These efforts will improve the accessibility of FMT and allow more physicians and patients to take advantage of its high success rate.

In addition to ongoing practical issues with FMT, the mechanisms explaining the success of the procedure are poorly understood, though it is widely accepted that the transferred fecal microbiota are responsible, directly or indirectly, for patient recovery.

Several studies have now demonstrated that following FMT, the fecal microbiota of patients rapidly shifts to a composition more similar to that of the donor (Khoruts et al., 2010; Shahinas et al., 2012; Hamilton et al., 2013; van Nood et al., 2013; Song et al., 2013; Weingarden et al., 2014; Seekatz et al., 2014; Fuentes et al., 2014). Specifically, fecal bacterial communities prior to FMT in R-CDI patients are typically dominated by members of the *Proteobacteria* phylum. Within a few days following transplant, however, the *Bacteroidetes* and *Firmicutes* phyla become dominant. These phyla, along with the *Actinobacteria*, are generally thought to dominate the fecal microbiota of healthy individuals (Zoetendal et al., 2008; Arumugam et al., 2011), and indeed are the most common phyla in fecal samples from healthy donors used in FMT (Shahinas et al., 2012; Hamilton et al., 2013; van Nood et al., 2013; Song et al., 2013; Weingarden et al., 2014; Seekatz et al., 2014; Fuentes et al., 2014). It is thought, therefore, that the fecal microbiota of the healthy donor engraft in the colon of the transplanted patient and rapidly replace the dysbiotic, *Proteobacteria*-dominated population, which prevents reinfection with *Clostridium difficile*. However, engraftment of donor microbes, versus re-growth of a patient's own native populations, has not been clearly demonstrated. Furthermore, it has been suggested that the new fecal community remains unstable for weeks after FMT (Song et al., 2013), implying that further study on how engraftment may be defined and what happens to patient fecal communities after the transplant is required.

In addition to investigating differences between donor and patient fecal populations, some work has attempted to identify specific bacteria responsible for recovery. Petrof and colleagues demonstrated that inoculation with 33 bacterial isolates

recovered from healthy human stool prevented recurrence of CDI in two patients (Petrof et al., 2013). Another group demonstrated that a cocktail of six species cured persistent infection in mice (Lawley et al., 2012). Meanwhile, recent work by Buffie et al. has found that a single organism, *Clostridium scindens*, one of few characterized 7α-dehydroxylating bacteria in the human gut, can increase survival in mice infected with *C. difficile*, although a consortium of four bacteria was more effective (Buffie et al., 2014). This work in particular emphasizes that the mechanisms behind FMT may not simply be "colonization resistance," or the ability of the native colonic microbiota *en masse* to outcompete *C. difficile* for nutrients or space (Hopkins and Macfarlane, 2003; Khoruts and Sadowsky, 2011; Britton and Young, 2012), but may in fact be related to specific functions of a healthy microbiota which directly inhibit the pathogen.

To emphasize the idea that specific metabolic functions of the microbiota may control *C. difficile* pathogenesis, evidence is emerging that certain commensal bacteria can in fact promote CDI. The metabolism of host mucin by the symbiont *Bacteroides thetaiotomicron* in gnotobiotic mice releases carbohydrates such as succinate which can be metabolized by *C. difficile* to promote the pathogen's expansion (Ng et al., 2013). These same authors later showed that succinate is increased in antibiotic-treated conventional mice, and that *C. difficile* which lacks the ability to metabolize succinate is at a competitive disadvantage compared to wild-type *C. difficile* in mice (Ferreyra et al., 2014). These findings suggest that altered carbohydrate metabolism by intestinal microbiota following antibiotic treatment permits growth of *C. difficile*, and implies that restoration of normal carbohydrate metabolism could be a mechanism behind FMT.

An accurate understanding of the mechanisms of FMT is critical not only to improve treatment of CDI, but also to assess whether FMT may be useful in treating other conditions. Currently, it is known that the fecal microbiota are altered or dysbiotic compared to healthy individuals in a vast number and variety of diseases, including inflammatory bowel disease (Tamboli et al., 2004; Swidsinski et al., 2005; Frank et al., 2007; Knights et al., 2014), atherosclerosis (Karlsson et al., 2012), metabolic syndrome (Ley et al., 2006; Turnbaugh et al., 2009; Karlsson et al., 2013; Joyce et al., 2014), type 1 diabetes mellitus (Brown et al., 2011; Giongo et al., 2011, de Goffau et al., 2013; Kostic et al., 2015), and autism (Williams et al., 2011). Because FMT can significantly alter the fecal microbiota of individuals with CDI, it is possible that the use of FMT in patients with these other conditions may shift fecal communities towards those of healthy donors, and therefore may potentially correct or ameliorate the associated disease.

One such disease in which FMT is being investigated is inflammatory bowel disease (IBD). An early case series of patients with ulcerative colitis, a subset of IBD, indicated that FMT could completely abrogate symptoms in patients refractory to other forms of therapy (Borody et al., 2003). A recent randomized controlled trial in ulcerative colitis patients, however, found no significant difference in remission with FMT compared to controls, although the patients who did respond to FMT had greater alteration in their fecal microbial communities, remaining similar to donor communities for 12 weeks (Rossen et al., 2015). FMT may therefore be promising for patients with ulcerative colitis, but its efficacy seems to be lower than in patients with CDI, possibly related to poorer engraftment of donor microbiota. A clearer definition of engraftment along with an understanding of how engraftment occurs, or knowledge of the

mechanisms behind FMT leading to the design of novel, non-FMT therapeutics, could drastically improve our treatment of IBD.

Another promising target for FMT is metabolic syndrome. Metabolic syndrome is a suite of risk factors including central obesity, elevated blood glucose, hypertension, and elevated blood triglycerides which can lead to cardiovascular disease, stroke, type 2 diabetes, and other serious conditions (Alberti et al., 2005). As in IBD, substantial work has revealed differences in fecal microbiota between obese and lean individuals (Ley et al., 2006; Turnbaugh et al., 2009; Karlsson et al., 2013; Joyce et al., 2014), and some studies have also demonstrated that transfer of microbes from obese animals or humans is sufficient to induce obesity in gnotobiotic animals (Turnbaugh et al., 2006; Ridaura et al., 2013; Duca et al., 2014). A small study found that transplant of fecal microbiota from lean individuals into patients with metabolic syndrome significantly increased peripheral insulin sensitivity, while autologous transplant with the patient's own feces did not (Vrieze et al., 2012). These findings strongly suggest that FMT may be a useful therapy for metabolic syndrome, although more work is still needed to demonstrate efficacy in a larger patient cohort and over longer periods of time.

In summary, *Clostridium difficile* infection is a widespread and often difficult to treat disease. Increased understanding of the basic biology of the pathogen, particularly its survival in the environment as spores and subsequent germination and outgrowth in the colon of susceptible individuals, is likely to be critical for the development of novel therapies for this infection. While fecal microbiota transplantation has emerged as a powerful therapy for CDI, particularly for those individuals who are refractory to standard antibiotic treatment, the procedure remains poorly understood. As a result, we

are limited in our ability both to design new treatments for this infection and to expand the use of FMT into other diseases. In this thesis, we present our findings into the mechanisms of FMT and subsequent rational design of novel therapeutics for *Clostridium difficile* infection.

Chapter 2

Dynamic Changes in Short- and Long-Term Bacterial Composition Following Fecal Microbiota Transplantation for Recurrent *Clostridium difficile* Infection[*]

[*] Reprinted from *Microbiome*. Alexa Weingarden, Antonio Gonzalez, Yoshiki Vazques-Baeza, Sophie Weiss, Gregory Humphry, Donna Berg-Lyons, Dan Knights, Tatsuya Unno, Aleh Bobr, Johnthomas Kang, Alexander Khoruts, Rob Knight, and Michael J. Sadowsky. "Dynamic changes in short- and long-term bacterial composition following fecal microbiota transplantation for recurrent *Clostridium difficile* infection." © **Weingarden et al.; licensee BioMed Central. Originally published in *Microbiome*. 2015. 3:10.**

SUMMARY

Fecal microbiota transplantation (FMT) is an effective treatment for recurrent *Clostridium difficile* infection (CDI) that often fails standard antibiotic therapy. Despite its widespread recent use, however, little is known about the stability of the fecal microbiota following FMT. Here we report on short- and long-term changes, and provide kinetic visualization of fecal microbiota composition in patients with multiply recurrent CDI that were refractory to antibiotic therapy and treated using FMT. Fecal samples were collected from four patients before and up to 151 days after FMT, with daily collections until 28 days and weekly collections until 84 days post-FMT. The composition of fecal bacteria was characterized using high throughput 16S rRNA gene sequence analysis, and compared to microbiota across body sites in the Human Microbiome Project (HMP) database, and visualized in a movie-like, kinetic format. FMT resulted in rapid normalization of bacterial fecal sample composition from a markedly dysbiotic state to one representative of normal fecal microbiota. While the microbiome appeared most similar to the donor implant material one day post-FMT, the composition diverged variably at later time points. The donor microbiota composition also varied over time. However, both post-FMT and donor samples remained within the larger cloud of fecal microbiota characterized as healthy by the HMP. Dynamic behavior is an intrinsic property of normal fecal microbiota, and should be accounted for in comparing microbial communities among normal individuals and those with disease states. This also suggests that more frequent sample analyses are needed in order to properly assess success of FMT procedures.

INTRODUCTION

Fecal microbiota transplantation (FMT) has emerged in recent years as a highly effective treatment for refractory *Clostridium difficile* infection (CDI) that cannot be cured with antibiotics alone (Khoruts and Weingarden, 2014). The procedure leads to prompt engraftment of donor microbiota, attainment of donor-like bacterial diversity, and normalization of the overall microbial community structure (Khoruts et al., 2010; Shahinas et al., 2012; Hamilton et al., 2012; van Nood et al., 2013; Weingarden et al., 2014; Seekatz et al., 2014; Fuentes et al., 2014). However, existing data characterizing long-term stability of engrafted microbiota are limited. One recent study suggests the microbiota of patients after FMT may not fully recover until 16 weeks after the procedure (Song et al., 2013). This type of analysis, however, is complicated by the fact that the microbial communities are intrinsically dynamic, and affected by daily fluctuations in the host's diet, activities, and health (Dethlefsen and Relman, 2011). In addition, multiple fixed host factors, such as different states of immune competence, genetics, or gastrointestinal anatomy, likely also affect the composition, stability, or resilience of colonic microbiota (Bäckhed et al., 2005; Lupp et al., 2007; Koren et al., 2012; Lozupone et al., 2013; Kawamoto et al., 2014). Therefore, it is unclear whether divergence in post-FMT microbiota from that of donor implant material represents continued recovery, or whether these temporal changes are a general characteristic of host-associated gut microbiota in a changing host environment.

Here we describe both short- and long-term dynamic changes of fecal bacterial composition in four patients following FMT. All patients received microbiota from the

same pre-qualified donor according to the standardized FMT protocol we described previously (Hamilton et al., 2012). Three patients received freshly prepared microbiota and one patient received microbiota that had previously been frozen. We compared pre- and post-FMT fecal microbial communities from these patients, as well as pre-FMT communities from 10 additional patients with multiply recurrent CDI (R-CDI), to the sequences of normal subjects described in the Human Microbiome Project (Turnbaugh et al., 2007). In addition, we compared temporal changes in fecal bacterial composition in recipients following FMT to temporal changes observed within samples from the donor.

MATERIALS AND METHODS

Patients and donors

All patients suffered from multiply recurrent CDI refractory to standard antibiotic therapies. A single standard donor was used in the preparation of all fecal microbiota material as described previously (Hamilton et al, 2012). The Institutional Review Board at the University of Minnesota approved prospective collection of fecal specimens and their analysis. All patients satisfied the inclusion criteria for the FMT within our program, which included at least two spontaneous recurrences of CDI within a month of discontinuation of antibiotics and failure of at least one advanced antibiotic regimen such as a vancomycin pulse/taper protocol or vancomycin treatment followed by administration of rifaximin or fidaxomicin for 2-3 weeks. The specific clinical characteristics of patients involved in this study are summarized in Table 2.1.

Fecal microbiota transplantation

FMT was done using a standardized preparation of concentrated fresh or frozen fecal bacteria via colonoscopy as previously described (Hamilton et al., 2012). All patents were treated with oral vancomycin, 125 mg four times daily, until two days prior to the procedure (Hamilton et al., 2012). The day before the procedure, patients received a polyethylene glycol-based colonoscopy prep (GoLYTELY® or MoviPrep®) to remove residual antibiotics and fecal material. Donor fecal microbiota was placed into the terminal ileum and/or cecum via the biopsy channel of the colonoscope. A total of 17 donor samples from the same individual were used in these studies. The CD1-CD4 donor

samples were given to patients CD1-CD4, respectively. Patients CD1, CD3, and CD4 received freshly prepared fecal microbiota, while patient CD2 received a previously frozen preparation of fecal microbiota, all from the same standardized, anonymous donor.

Sample collection

Fecal samples were collected at home by the patients using swabs to sample feces deposited into a toilet hat immediately after production, and stored frozen at approximately -20^{o}C. Samples were subsequently transferred to the laboratory and stored at -80^{o}C until used. Donor samples for DNA extraction were collected during processing of material for FMT and stored frozen at -80^{o}C until used. Samples from patients CD1- 4 were obtained prior to FMT and between 1 to 151 days post-FMT, with daily collection until day 28, and weekly collection until day 84. Fecal material prior to FMT was obtained from patients CD5-CD14.

DNA extraction

DNA was extracted from donor and recipients' pre- and post-FMT fecal samples using MOBIO PowerSoil DNA extraction kits (MOBIO, Carlsbad, CA), according to the manufacturer's instructions. Fecal DNA concentrations were measured using a QuBit DNA quantification system (Invitrogen, Carlsbad, CA).

PCR amplification

Extracted DNA was amplified using the EMP standard protocols at http://www.earthmicrobiome.org/ following the recommendations of Caporaso et al.,

2012. Briefly, F515/R806 primers were used, with 12-base Golay codes introduced on the 806 end to provide unique sample indices. Cycling and annealing conditions were as previously described (Caporaso et al., 2012).

DNA sequencing

DNA sequencing was performed as previously described (Caporaso et al., 2012) on an Illumina MiSeq platform using 2 x 150 bp paired-end reads and the Illumina v3 reagent chemistry.

Sequence processing and analysis

Sequence data was processed and analyzed using QIIME (Caporaso et al., 2010) according to the Illumina demultiplexing and processing protocol (Caporaso et al., 2012) and current quality-filtering recommendations (Bokulich et al., 2013), using the 1.8.0 pipeline and the default parameters in split_libraries_fastq.py. After quality control and demultiplexing, we picked close references at 97% similarity against the 97% similarity Greengenes database (McDonald et al., 2012) version 13_8. All further analyses were performed at a rarefied depth of 5,000 reads/sample. EMPeror (Vazquez-Baeza et al., 2013) was used for data visualization of BIOM-format (McDonald et al., 2012) OTU tables. OTU analyses were performed by clustering at the 97% level with UCLUST (Edgar, 2010), and data were integrated with the HMP dataset according to the protocols used for similar previous meta-analyses (Koren et al., 2012; Lozupone et al., 2013). Sequences were analyzed by using both weighted and unweighted UniFrac (Lozupone et al., 2013), followed by principal coordinate analysis (Caporaso et al., 2010). Data were

visualized using Phinch. The Phinch program provides an easy-to-use, browser-based, platform to visualize contingency tables along with their sample metadata (Bik et al., manuscript in preparation, https://github.com/PitchInteractiveInc/Phinch).

Analysis of microbiome normalization and dynamic range

For each set of post-transplant patient samples we assessed the similarity of that set to the set of reference samples from the donor (2,000 reads/sample). To reduce noise and compare patient samples along only relevant dimensions in UniFrac distance space, we applied principal coordinates analysis (PCoA) to the unweighted UniFrac distance matrix containing only the post-transplant and donor samples for that donor-patient pair, then recalculated the distances using only the first *n* principal coordinates axes required to explain at least 80% of the variation in the distance matrix. An 80% cutoff was chosen to balance bias and overfitting. Distances were recalculated using Euclidean distances between points in PCoA space in order to convert PCoA coordinates to a distance matrix. The empirical p-values for the 'normality' were obtained by comparing the mean distance between patient and donor samples to the histogram of within-donor distances (generated using all samples from a given donor by enumerating the pairwise distance between those samples). The empirical p-values for the 'dynamic range' (stability) were obtained by comparing the mean distance within patient samples to the histogram of within-donor distances. These analyses were also performed using alternative parameters including, weighted UniFrac, Jensen-Shannon, root Jensen-Shannon, and Bray-Curtis.

RESULTS

Bacterial composition of fecal samples from patients with recurrent CDI becomes healthy and donor-like following FMT

Four patients (CD1-CD4) with recurrent CDI were treated with FMT using material obtained from a single donor but from different time points, and fecal samples were collected from these patients before and after the procedure as well as from the donor at times of donation. Bacterial communities from these fecal samples were characterized by sequencing the V4 region of the 16S rRNA gene. Following trimming and quality filtering from a total of 12,536,492 sequences, we randomly subsampled to 5,000 sequences/sample in order to normalize read depth across all samples. All further analyses were performed using this rarefied read depth.

To better understand changes in bacterial communities following FMT, we compared the bacterial composition of patient fecal samples to those of microbial communities from various body sites from the 252 healthy individuals characterized in the Human Microbiota Project (HMP) (Turnbaugh et al., 2007) (Fig. 2.1) using unweighted UniFrac (Lozupone et al., 2013) followed by principal coordinates analysis (PCoA) (Caporaso et al., 2010) (see Movie Supplement). The composition of pre-FMT fecal samples from patients CD1-CD4 and 10 additional patients with recurrent CDI was distinct from both fecal samples from healthy individuals and microbial communities at other body sites, including mouth, vagina, and skin, demonstrating severe alterations in pre-FMT communities compared to healthy fecal communities as has been previously shown (Hamilton et al., 2013; van Nood et al., 2013). In contrast, microbial communities

from the donor fell within the range of healthy fecal samples. Using an animated visualization of FMT-associated changes in patients' fecal microbial communities, we observed rapid and dramatic shifts after FMT towards the communities found in the feces of healthy individuals and of the original donor (see Movie Supplement).

Fecal microbial communities remain dynamic following FMT

To more closely examine temporal changes in recipient fecal samples following FMT, we analyzed fecal microbial communities from patients CD1-CD4 and donor, as well as from 10 additional donor samples, using weighted and unweighted UniFrac (Lozupone et al., 2005) followed by PCoA (Caporaso et al., 2010). This analysis demonstrated that fecal bacterial communities continued to undergo compositional fluctuation following FMT (Fig. 2.2A and Fig. 2.3; individuals OTUs listed in Supplementary Table).

To determine whether this dynamic range of post-FMT microbial composition fits within the range seen across healthy individuals, we also compared communities in our samples to those in the HMP via weighted UniFrac and PCoA (Fig. 2.2B). Again, fecal microbial communities prior to FMT were highly distinct from healthy fecal microbial communities, and following the procedure these communities more closely resembled those of healthy individuals. Similar to the comparison with donor communities above, fecal microbial communities of recurrent CDI patients following FMT shifted within the cluster of communities from healthy individuals.

Rapid and substantial changes to Enterobacteriales in feces following FMT

While overall fecal microbial communities were dramatically altered following FMT, we also examined the effects of the procedure on the abundance and dynamics of individual bacterial taxa within the four original CDI patients. As shown previously (Khoruts et al., 2010; Shahinas et al., 2012; Hamilton et al., 2012; van Nood et al., 2013; Weingarden et al., 2014; Seekatz et al., 2014; Fuentes et al., 2014), the relative abundance of bacterial phyla in patient fecal samples shifted substantially following FMT, with relative decreases in *Proteobacteria* and relative increases in *Bacteroidetes* and *Firmicutes* (Fig. 2.4). These *Proteobacteria* are primarily the order *Enterobacteriales*, which were also substantially decreased in relative abundance following FMT (Fig. 2.5A).

We focused on these changes by examining the relative abundance of *Enterobacteriales* alone in each patient before and after FMT. The relative abundance of this taxon ranged from 44 to 82% in all four patient samples prior to FMT, and rapidly dropped to undetectable levels within one week after the procedure. Moreover, abundance of this taxon remained low at 26 days after FMT, the latest time point shared by all four patients (Fig. 2.5A), although other members of the *Proteobacteria* remain detectable if decreased in relative abundance (Fig. 2.4). In addition, we generated individual value control charts based on the average abundance of this taxon in recurrent CDI patients. Compared to relative abundance, these control charts displayed the expected variation of the abundance of *Enterobacteriales* in these fecal samples. In all patients, the abundance of *Enterobacteriales* was above the expected variation (i.e. more than three standard deviations above the mean relative abundance [the standard upper control limit, or UCL] of this order across all samples) prior to FMT, and rapidly fell

below the upper control limit within 1-2 days after the procedure (Fig. 2.5B). These results suggest that the relative abundance of *Enterobacteriales* significantly decreased in all patients soon after FMT to levels similar to donor samples, and remained within a statistically expected range for the duration of sample collection (up to 151 days post-FMT).

Post-FMT communities are initially similar to donor samples but can later diverge

Next, we compared fecal microbial communities within each patient over time to that of the initial donor sample. We generated heat maps based on Pearson correlations between every sample within a given patient set, including respective donor samples and samples from 10 additional pre-FMT patients (Fig. 2.6A). This analysis revealed that while microbiota in samples from patients after FMT quickly became similar to microbiota in donor samples, the similarity of samples taken at later time points after FMT fluctuated.

To further investigate how fecal microbial communities in these patients correlate to donor communities, we examined Pearson and Spearman correlations between donor and patient samples, which were common to each patient (pre-FMT samples and those up to 26 days post-FMT; Fig. 2.6B and C and Fig. 2.7). While fecal microbial communities from patients before FMT were highly distinct from those in the donor, fecal microbial communities from samples one day after the procedure were highly correlated to donor communities via both Pearson and Spearman analyses in all patients. After the initial time point after FMT, the Pearson correlation values of patient to donor samples were highly variable within and across patients, although Spearman correlations remained high for

three patients. To examine whether this variation is similar in healthy individuals, we determined Pearson and Spearman correlations within the four donor samples used in FMT, as well as eight additional donor samples from the same individual as a control. Results of this analysis revealed that donor microbiota also changed over time (Fig. 2.6D). These findings suggested that the level of variability seen across patient post-FMT fecal microbial communities was within the range of normal microbiota behavior in a healthy individual.

Normalization and dynamic range of post-FMT patient fecal microbial communities are similar to donor communities

Because of the observed variability in later post-FMT patient fecal communities relative to single donor communities, we compared the communities of these patient samples to an expanded set of 17 samples taken from the same donor. We generated two metrics to evaluate the relationships between these communities: normalization and dynamic range (stability). Normalization refers to the mean between-sample distance for each set of patient samples versus the set of donor samples, while dynamic range is the mean distance between each sample within a single patient set. Effectively, the normality of a post-FMT patient sample set is a measure of how similar it is to the donor (healthy) sample set, while dynamic range is a measure of variability within a given patient sample set. We found that neither the normalization nor the dynamic range of any post-FMT patient sample set was significantly different than the donor set following analysis using unweighted UniFrac (Table 2.2). This suggested that although fecal microbial communities of patients post-FMT do not remain identical to the donor, they nonetheless

fall within expected parameters relative to the healthy donor. Similar results were obtained when these analyses were repeated with other parameters, including weighted UniFrac, Jensen-Shannon and root Jensen-Shannon, and Bray-Curtis (data not shown).

DISCUSSION

It is now well understood that the fecal microbiota change substantially following FMT, typically shifting to fecal microbial communities more similar to those of the donor after transplant (Khoruts et al., 2010; Shahinas et al., 2012; Hamilton et al., 2012; van Nood et al., 2013; Weingarden et al., 2014; Seekatz et al., 2014; Fuentes et al., 2014). Here we show that these communities shift away from a dysbiotic state towards a composition that is representative of fecal microbial communities from hundreds of healthy individuals, collected in the HMP (Turnbaugh et al., 2007). Similarly to previous studies (Hamilton et al., 2012; van Nood et al., 2013; Weingarden et al., 2014; Seekatz et al., 2014; Fuentes et al., 2014), the dysbiotic state in these patients with multiply recurrent CDI is characterized by a large expansion of *Proteobacteria* (primarily members of the order *Enterobacteriales*, which contains the family *Enterobacteriaceae*), and FMT is associated with reemergence of dominance by members of the *Bacteroidetes* and *Firmicutes* phyla.

Analysis of multiple donor and post-FMT samples demonstrates the dynamic behavior of fecal microbial communities over time. Both donor and recipient samples are characterized by highly dynamic shifts that nonetheless remain within the compositional range of normal fecal microbiota. This observation is consistent with known rapid responsiveness of the fecal microbiome to environmental inputs, such as dietary variations (David et al., 2014), and drifts in microbiota composition over time in healthy individuals (Faith et al., 2013).

The dynamic nature of intestinal microbiota is an intrinsic property, which should be taken into account when considering how therapeutic interventions, including FMT, impact its composition over time. In long-term post-FMT follow-up, Song and colleagues also noted dynamic changes in the fecal microbiome of R-CDI patients up to 16 weeks post-FMT (Song et al., 2013). These investigators concluded that the fecal microbiome of post-FMT patients did not fully recover over this time, despite clinical recovery. Indeed, we observed divergence of microbiome in some of the patients away from the original implanted material over time. However, analysis of multiple donor samples showed that this movement is within the same dynamic range observed in the donor's fecal microbiome. We therefore conclude that the dynamic behavior of microbiota needs to be taken into account in making comparisons between individuals, and should become an integral part of analysis of the success of FMT.

Three of the recipients in this study received freshly prepared microbiota, while one received frozen/thawed preparation. Use of frozen microbiota preparations is increasing in clinical practice (Youngster et al., 2014), and its equivalency has not been rigorously established in randomized clinical trials. The ability to store microbiota allows the most up-to-date testing of the donor and fecal material for infectious pathogens, as some of the current tests may take several weeks to complete. Therefore, ability to preserve donor microbiota long-term is critical for its development as a therapeutic agent in clinical practice. Our results here, although limited in the number of patients, demonstrate indistinguishable behavior of fresh and frozen/thawed microbiota preparation.

The patients in this study did not have any significant gastrointestinal co-morbidities. However, a significant proportion of patients with recurrent CDI have underlying inflammatory bowel disease, take potent immunosuppressive medications, or have multiple other medical problems (Hamilton et al., 2012; Kelly et al, 2014). The importance of these host factors in contributing to microbiota behavior is currently unknown, but is a subject of great interest (Gevers et al., 2014). Understanding these influences will require analysis of multiple samples. Recently, Fuentes and colleagues (Fuentes et al., 2014) reported that some specific microbial groups and interactive networks are likely to be very important for the maintenance of microbiota in healthy individuals. However, although there is a great deal of effort focused on discovery of compositional differences in microbiota between normal subjects and individuals with different gastrointestinal and medical conditions, the dynamic behavior of fecal microbiota constitutes another dimension that may distinguish these cases. Thus, predictors of stable or dysbiotic intestinal microflora may also change over time. Further detailed studies of dynamic behavior of post-FMT microbiota may improve our understanding of causal connections between microbial communities and different disease states.

The fecal microbiota of patients with R-CDI continues to undergo change after FMT is performed, though these changes appear to fall within the range of normal variation of healthy individuals over time. Dynamic behavior is an intrinsic property of normal fecal microbiota, and should be accounted for in comparing microbial communities among normal individuals and those with disease states.

Table 2.1 - Clinical metadata of patients used in this study.

Patient	Age	Gender	Trigger Event	Trigger Antibiotic	Duration of RCDI prior to FMT (months)	Co-morbidities; History of GI Surgery	History of Treatment of CDI with Metronidazole	History of Treatment of CDI with Vancomycin	History of Treatment of CDI with Rifaximin	History of Treatment of CDI with Fidaxomicin	History of Trreatment with Nitazoxanide
CD1	39	F	Antibiotics with Caesarian section	N/A	18	None	Yes	Yes	Yes	No	Yes
CD2	52	M	Sinusitis	N/A	5	None	Yes	Yes	Yes	No	No
CD3	55	M	Antibiotics with a dental procedure	N/A	18	Immunoglobulin deficiency secondary to administration of rituximab; s/p cholecystectomy	Yes	Yes	Yes	No	No
CD4	60	F	Diverticular abcess	Ciprofloxacin and Metronidazole	7	None	Yes	Yes	No	No	No
CD5	83	F	Sepsis (*Proteus mirabilis*)	N/A	8	None	Yes	Yes	No	No	No
CD6	71	M	N/A	N/A	7	Diverticulosis	Yes	Yes	No	No	No
CD7	66	F	Antibiotics with spinal surgery	N/A	3	S/P Billroth II gastrectomy; Diverticulosis	Yes	Yes	No	No	No
CD8	72	M	Pneumonia	Trimethoprim/sulfamethoxazole; vancomycin;	13	S/P heart transplant; Liver cirrhosis due to	Yes	Yes	No	No	No

				levofloxacin; meropenem		hepatitis B; Diverticulosis					
CD 9	65	F	Vaginal infection	Clindamycin	7	S/P appendectomy	Yes	Yes	Yes	No	No
CD 10	47	F	Upper respiratory tract infection	Clindamycin	3	S/P cholecystectomy; Lymphocytic colitis	Yes	Yes	Yes	No	No
CD 11	36	M	N/A	N/A	8	None	Yes	Yes	No	No	No
CD 12	52	F	Antibiotics with hysterectomy	N/A	5		Yes	Yes	Yes	Yes	No
CD 13	56	F	Antibiotics with knee replacement	N/A	3	None	Yes	Yes	No	Yes	No
CD 14	53	F	Urinary tract infection	Ciprofloxacin	8	None	Yes	Yes	No	Yes	No

Table 2.2 - P-values of normalization and dynamic range of patient samples sets versus donor set.

Patient	CD1	CD2	CD3	CD4
Normalization	0.154	0.429	0.165	0.484
Dynamic Range	0.484	0.429	0.308	0.473

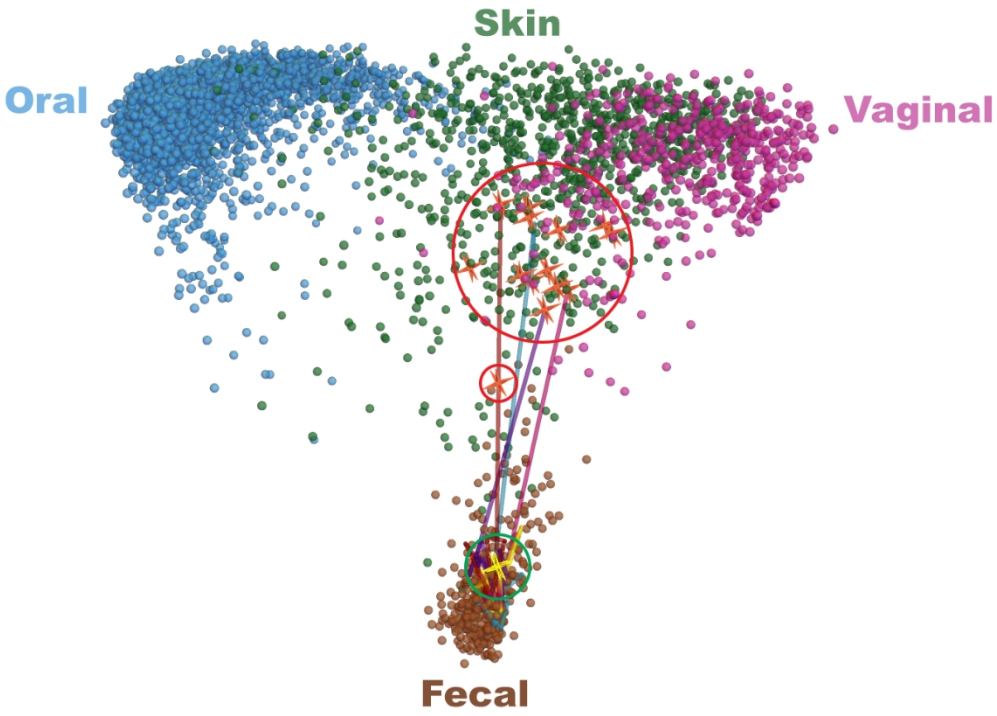


Figure 2.1 - Fecal bacterial communities of recurrent CDI patients shift towards HMP fecal bacterial communities after FMT. Red circles = pre-FMT patient samples; green circle = post-FMT patient samples; blue line = trajectory of patient fecal communities after FMT.

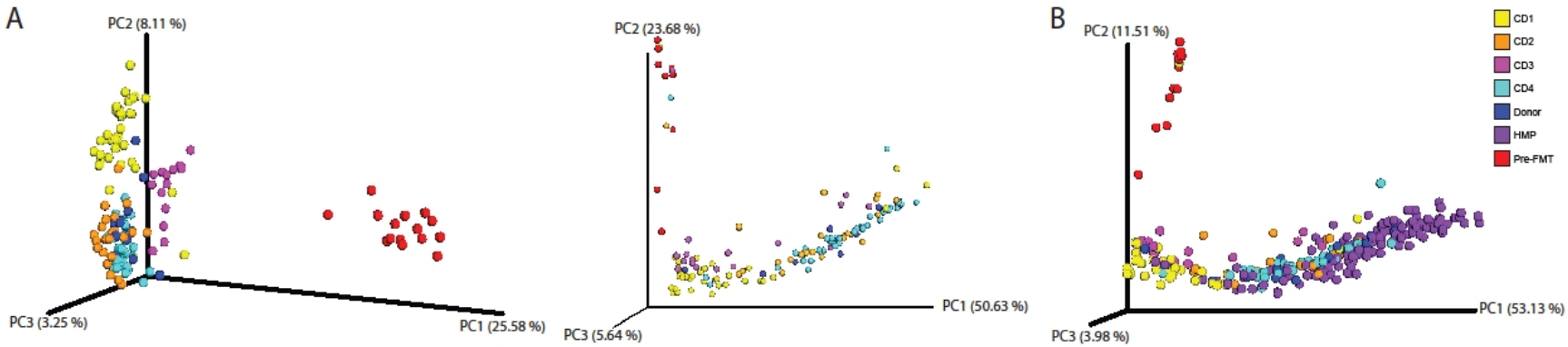


Figure 2.2 - Microbial communities shift following FMT. A) Unweighted (left) and weighted (right) UniFrac analyses followed by principal component analysis of bacterial communities of recurrent CDI patient fecal samples before (red) and after FMT and donor samples (blue). B) Weighted UniFrac analysis followed by principal component analysis of bacterial communities of patients before (red) and after FMT versus HMP fecal communities (purple). PC: principal component. Percentages represent percent variability explained by each principal component. Se key at right for colors associated with samples before FMT (Pre-FMT), from HMP and donor, and from patients after FMT (CD1-CD4).

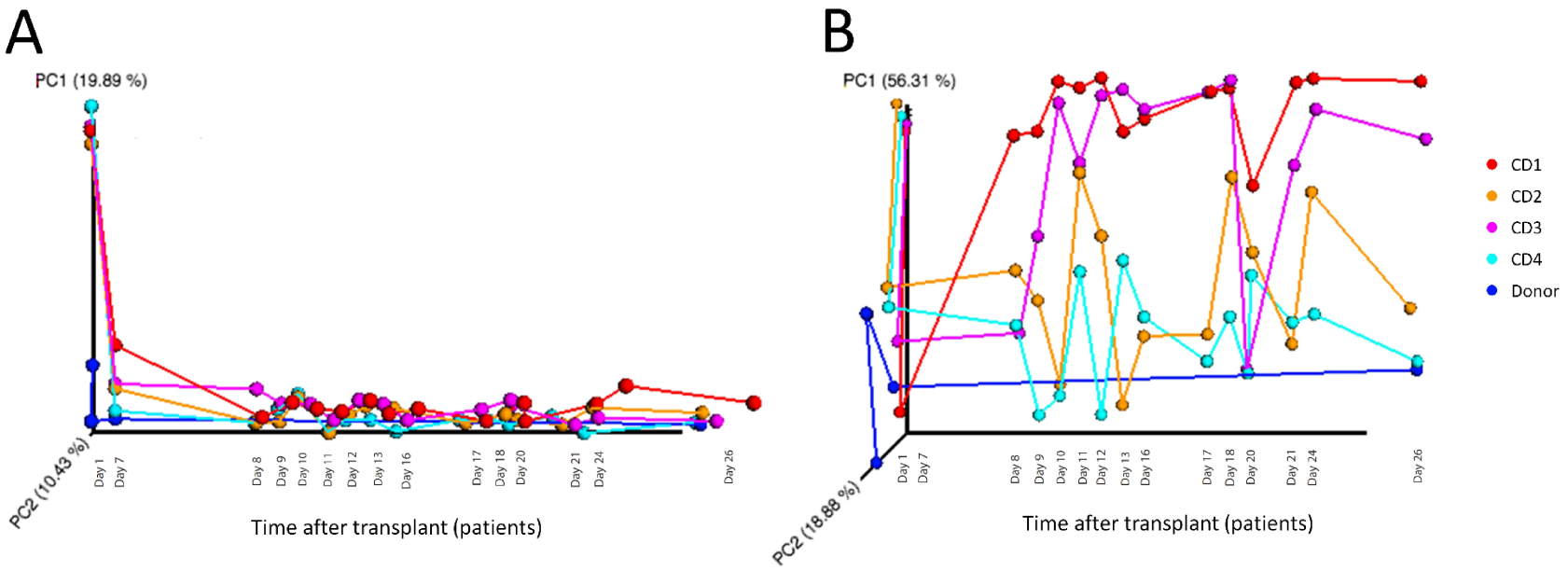


Figure 2.3 - Microbial communities remain dynamic after FMT. (A) Unweighted and (B) weighted UniFrac analyses, followed by principal component analysis of bacterial communities of recurrent CDI patient fecal samples, by time point after FMT and donor samples (blue). PC: principal component. Percentages represent percent variability explained by each principal component. See key at right for colors associated with samples from patients after FMT (CD1-CD4).

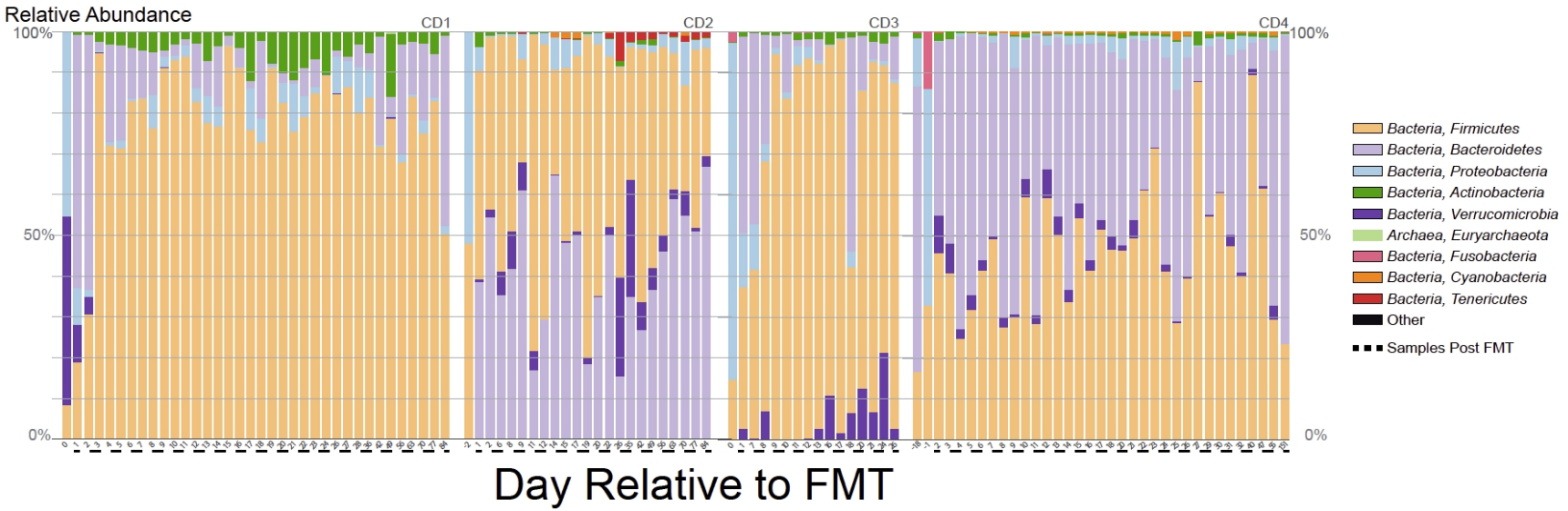


Figure 2.4 - Fecal microbial communities change after FMT. Relative abundance of sequences classified to the level of bacterial phyla before and after FMT in patient fecal samples. Samples after FMT indicated with dashed line. See key at right.

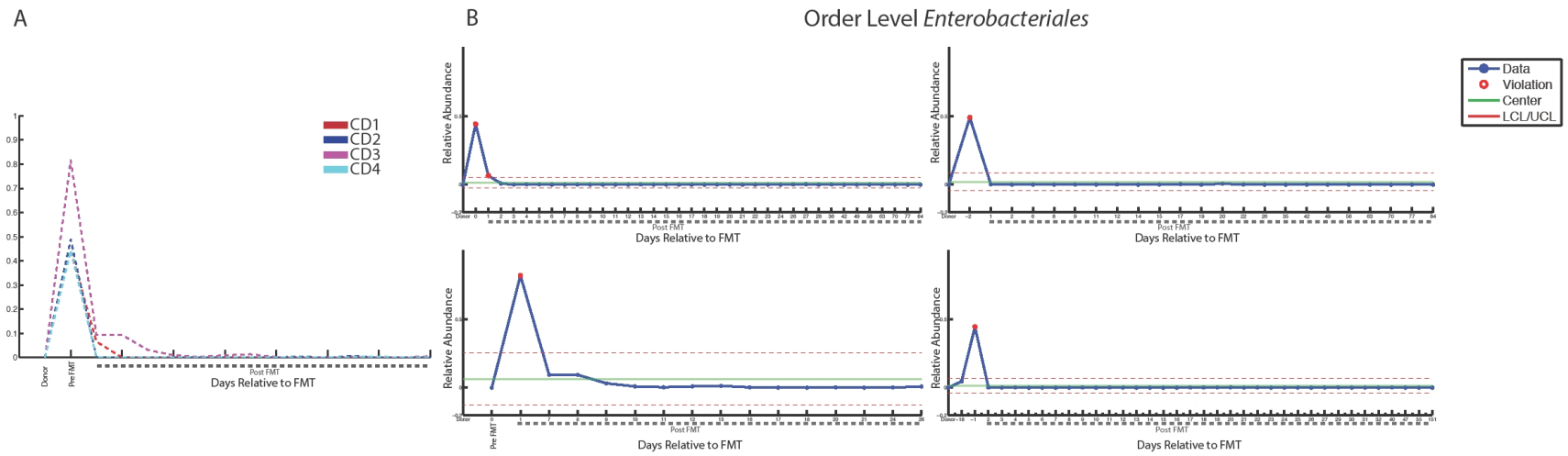


Figure 2.5 - Abundance of the order *Enterobacteriales* changes after FMT. A) Relative abundance of *Enterobacteriales* in donor and patient samples before and after FMT in samples common across all patients. B) Control charts of relative abundance of *Enterobacteriales* in donor (leftmost sample) and patient samples before and after FMT. Top left: patient CD1, top right: patient CD2, bottom left: patient CD3, bottom right: patient CD4. LCL: lower control limit; UCL: upper control limit; Center: mean relative abundance in all samples. LCL and UCL represent three standard deviations in relative abundance below and above the mean, respectively. Dashed lines indicate samples after FMT.

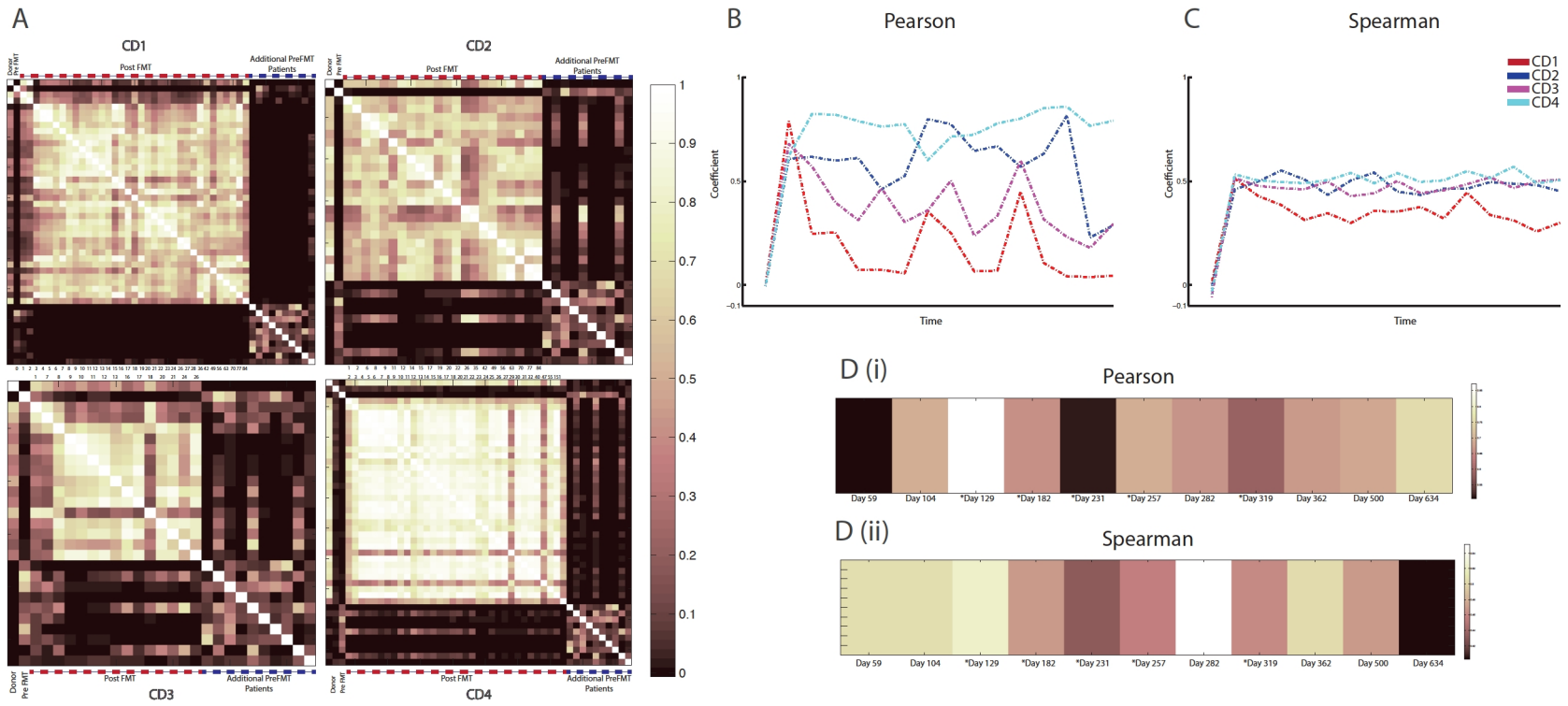


Figure 2.6 - Fecal communities of patients undergo shifts relative to donor after FMT. A) Heat map of Pearson correlation values between each sample within each patient set, corresponding donor, and 10 additional pre-FMT patient samples (far right). B) Pearson correlation values between donor sample and each patient sample. C) Spearman correlations between donor sample and each patient sample. D) Heat maps of Pearson (i) and Spearman (ii) correlation values between earliest donor sample and eleven subsequent

samples; days represent collection time of each sample versus earliest donor sample. CD1-4: patients 1-4. Dashed lines indicate samples after FMT.

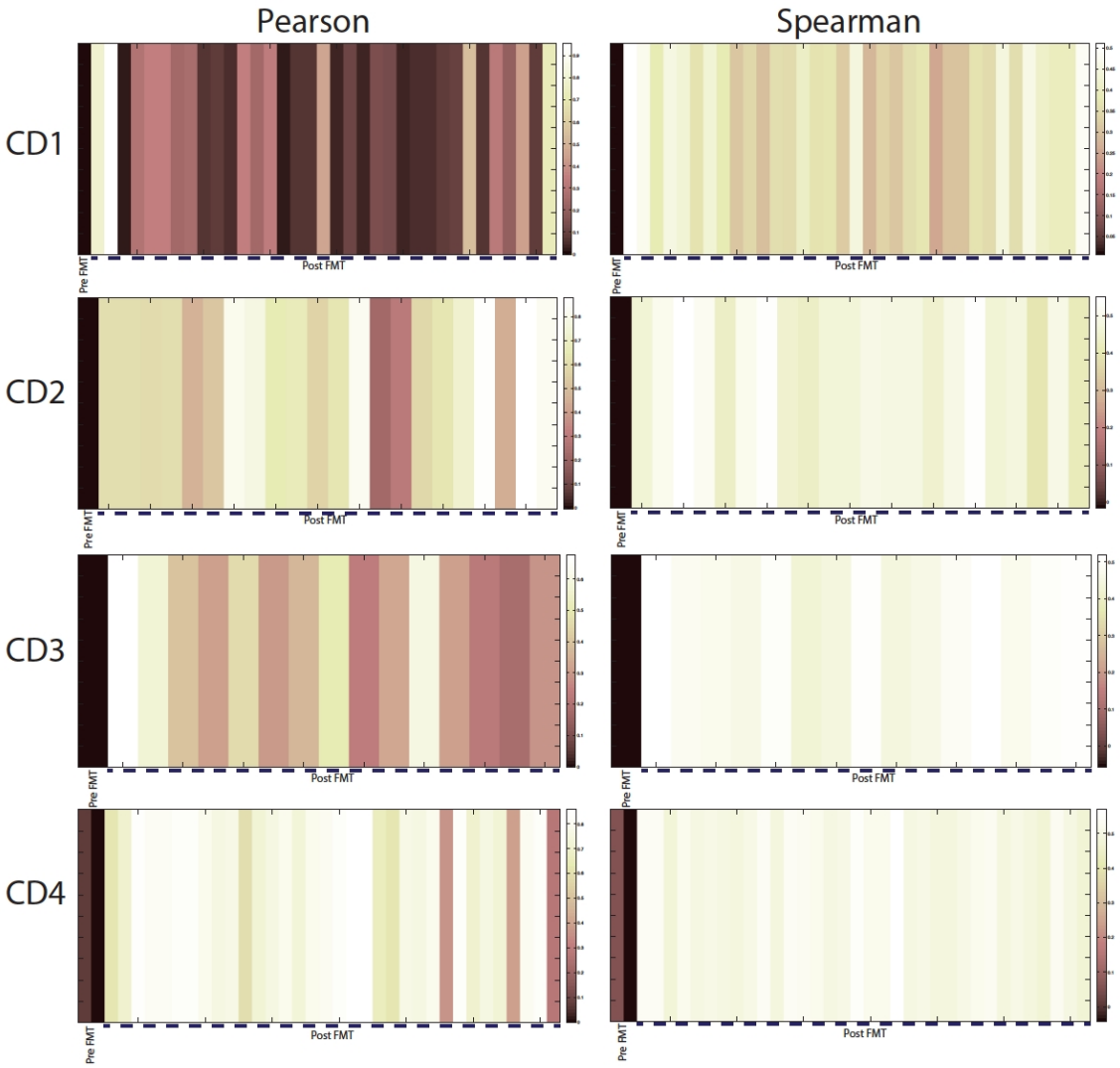


Figure 2.7 - Fecal communities of patients undergo shifts relative to donor after FMT. Heat maps indicating Pearson (left) and Spearman (right) correlation values between respective donor and pre- or post-FMT fecal microbial communities of patients.

Chapter 3

Microbiota Transplantation Restores Normal Fecal Bile Acid Composition in Recurrent *Clostridium difficile* Infection [†]

[†] Reprinted from *American Journal of Physiology, Gastrointestinal and Liver Physiology*. Alexa R. Weingarden, Chi Chen, Aleh Bobr, Dan Yao, Yuwei Lu, Valerie M. Nelson, Michael J. Sadowsky, and Alexander Khoruts. "Microbiota transplantation restores normal fecal bile acid composition in recurrent *Clostridium difficile* infection."

SUMMARY

Fecal microbiota transplantation (FMT) has emerged as a highly effective therapy for refractory, recurrent *Clostridium difficile infection* (CDI), which develops following antibiotic treatments. Intestinal microbiota play a critical role in the metabolism of bile acids in the colon, which in turn have major effects on the lifecycle of *C. difficile* bacteria. We hypothesized that fecal bile acid composition is altered in patients with recurrent CDI and that FMT results in its normalization. General metabolomics and targeted bile acid analyses were performed on fecal extracts from patients with recurrent CDI treated with FMT and their donors. In addition, 16S rRNA gene sequencing was used to determine the bacterial composition of pre- and post-FMT fecal samples. Taxonomic bacterial composition of fecal samples from FMT recipients showed rapid change to become similar to the donor after the procedure. Pre-FMT fecal samples contained high concentrations of primary bile acids and bile salts, while secondary bile acids were nearly undetectable. In contrast, post-FMT fecal fecal samples contained mostly secondary bile acids, as did non-CDI donor feces. Therefore, our analysis showed that FMT resulted in normalization of fecal bacterial community structure and metabolic composition. Importantly, metabolism of bile salts and primary bile acids into secondary bile acids is disrupted in patients with recurrent CDI, and FMT corrects this abnormality. Since individual bile salts and bile acids have both pro-germinant and inhibitory activities, the changes observed suggest that correction of bile acid metabolism is likely a major mechanism by which FMT results in a cure and prevents recurrence of CDI.

INTRODUCTION

Clostridium difficile infection (CDI) is currently one of the most common nosocomial infections in the US, affecting over 500,000 people annually in health care facilities and many more in the community (Ananthakrishnan, 2011; Miller et al., 2011; Khanna et al., 2012; Lessa et al., 2012; Chitnis et al., 2013). Unfortunately antibiotic therapy alone commonly fails to achieve a cure for this disease; in fact, the use of antibiotics is the primary risk factor for CDI and antibiotic treatment of CDI can perpetuate its recurrence (Borody and Khoruts, 2011; Miller et al., 2011; Surawicz and Alexander, 2011; Khanna et al., 2012). Approximately 20-30% of patients experience recurrence of the disease after antibiotic treatment of the initial infection (Kelly and Lamont, 2008; Louie et al., 2011). The risk of recurrence continues to rise with each relapse, and some patients ultimately develop a recurrent *C. difficile* infection syndrome (R-CDI), where the cycle of infections becomes indefinite (Borody and Khoruts, 2011; Surawicz and Alexander, 2011). Fecal microbiota transplantation (FMT), which achieves restoration of the normal composition of fecal bacteria, is a powerful emerging therapy that successfully treats over 90% of patients with R-CDI (Borody and Khoruts, 2011; Gough et al., 2011; van Nood et al., 2013). However, the mechanisms by which this transplanted microbiota prevents recurrence of CDI remain poorly understood.

One known function of the normal human gut microbiota, which may affect *C. difficile* physiology, is the metabolism of primary bile acids to secondary bile acids in the colon. The primary bile acids cholic acid and chenodeoxycholic acid are produced in the liver and conjugated to taurine or glycine to form bile salts before secretion into bile for

assisting lipid digestion in the small intestine (Hofmann and Hagey, 2008). While ~95% of secreted bile salts are reabsorbed from the small intestine via the enterohepatic circulation pathway, ~5% reach the colon, where by the actions of intestinal bacteria they are deconjugated and dehydroxylated at the C7 position to form deoxycholic and lithocholic acids, from cholic acid and chenodeoxycholic acid, respectively (Hofmann and Hagey, 2008). Considering the cytotoxic effects of antibiotics on microbiota, including normal gut microflora, it is unsurprising that a variety of antibiotics, including ciprofloxacin, neomycin, and β-lactams, significantly alter the fecal bile acid pool in both rodents and *in vitro* human fecal cultures, reducing the proportion of secondary relative to primary bile acids (Hashimoto et al., 1996; Carman et al., 2005; Toda et al., 2009; Giel et al., 2010). These findings suggest that antibiotic treatment is likely to substantially alter intestinal bacteria-mediated fecal bile acid metabolism in R-CDI patients.

Bile acids substantially affect germination and growth of *C. difficile*. The primary bile acid taurocholic acid, the taurine-conjugated form of cholic acid secreted by the liver, is a potent germinant of the organism, and is even used as a key component of *C. difficile* growth media (Wilson, 1983; Sorg and Sonenshein, 2008). Recently, a putative *C. difficile* bile acid germinant receptor was identified to be a protease CspC, and a *cspC* mutant of *C. difficile* had decreased pathogenicity in a hamster model (Francis et al., 2013). Notably, the secondary bile acids lithocholic and ursodeoxycholic acid have been shown to inhibit *C. difficile* germination *in vitro* (Sorg and Sonenshein, 2010). Fecal samples from mice treated with clindamycin, which have a significantly lower proportion of secondary bile acids compared to untreated mice, are better able to stimulate colony formation from *C. difficile* spores compared to controls, suggesting that secondary bile

acids may also inhibit germination and/or growth *in vivo* (Giel et al., 2010). These studies suggest that secondary bile acids in feces, which decrease following antibiotic treatment, may inhibit germination and/or growth of *C. difficile* in the colon.

To examine the influence of FMT on the gut microflora-mediated metabolism in R-CDI patients, we conducted general metabolomic and targeted bile acid analyses on fecal extracts from 12 R-CDI patients before and after treatment with FMT. We also analyzed the composition of fecal microbiota in these patients using high-throughput 16S rRNA gene sequencing. Here we demonstrate that restoration of normal bacterial composition of fecal microbiota is accompanied by rapid normalization of fecal bile acid composition in these patients. These results offer a potential mechanism by which FMT treats R-CDI and suggest novel pathways of therapeutics development for this frustrating clinical condition.

MATERIALS AND METHODS

Patients and donors

All patients recruited into this study had R-CDI syndrome and failed to clear the infection despite multiple rounds of antibiotic treatments (Table 3.1). Two standard donors were used in the preparation of fecal microbiota material as described previously (Hamilton et al., 2012). The Institutional Review Board at the University of Minnesota approved prospective collection of fecal specimens and their analysis (Protocol 1303M29781).

FMT procedure

The fecal microbiota were infused via colonoscopy as previously described (Hamilton et al., 2012). All patients took oral vancomycin until 2 days prior to the procedure. The patients received a polyethylene glycol-based colonoscopy prep the day prior to FMT (GoLYTELY® or MoviPrep). Fecal microbiota were prepared from one of two standard donors, selected as described previously using successive filtration to separate the microbial fraction from the rest of the fecal material (Hamilton et al., 2012). Ten patients received FMT which were frozen in 10% (v/v) glycerol and stored at -80°C until used as previously described (Hamilton et al., 2012), while 6 patients received freshly-processed (non-frozen) material. Recovery from CDI was defined by resolution of clinical diarrhea (≤ 3 bowel movements per day and normalization of stool consistency) over two months. All patients continue to be followed clinically since their FMT.

Sample collection

Fecal samples were collected by patients prior to FMT and at days 7 to 22 following FMT. All patients were actively taking vancomycin when pre-FMT samples were collected. Samples were stored at -80°C within 24 h of production until DNA extraction. An unprocessed portion of each donor sample was also collected at the time of donation and stored immediately at -80°C.

DNA extraction

DNA was extracted from each patient and donor sample (0.25 to 0.50 g) using the PowerSoil® DNA Isolation Kit (MO BIO, Carlsbad, CA) according to the manufacturer's instructions. Samples with high water content (Bristol stool scale types 5 to 7) were centrifuged at 12,000 RPM for 3 min to pellet solids, which were used for DNA extraction. Each sample was extracted in triplicate, and each replicate was eluted in 50 µl of 10mM Tris-HCl buffer (pH 8.0) and pooled. DNA concentrations of extracted samples were measured with a QuBit® DNA quantification system (Invitrogen, Carlsbad, CA) using QuBit high sensitivity assay reagents. All extracted DNA samples were stored at -20°C until amplification.

PCR amplification

Fecal DNA samples (25 ng) were used as template for PCR amplification of the V6 region of the 16S rRNA gene. Degenerate primer sets (Table 3.2) were designed with 6 bp Illumina index sequences on the 5' end of the reverse primer which were specific to each fecal DNA sample and allowed for multiplexed sequencing. Primers also contained Illumina PCR primer sequences (reverse primer) and Illumina TruSeq Universal Adapter

sequences (forward primers) for library creation. Triplicate reactions were electrophoresed on a 2% agarose gel, followed by extraction using the Qiaquick® Gel Extraction Kit (Qiagen, Valencia, CA), eluted in 30 µl of 10 mM Tris-Cl buffer, pH 8.0, and pooled. DNA concentrations were measured using the QuBit® DNA quantification system and high sensitivity assay reagents. Samples were stored at -20° C until pooled for sequencing.

DNA sequencing

Up to 24 equimolar aliquots of each product were pooled to give 3 samples of ~1 µg of DNA in 100 µL total volume. DNA concentration in the final pooled solutions was measured by using the Quant-IT™ PicoGreen quantitation system (Invitrogen, Carlsbad, CA). Amplicon size analysis was done using an Agilent DNA 1000 chip and a 2100 BioAnalyzer (Agilent, Santa Clara, CA). Sequencing was performed at the University of Minnesota BioMedical Genomics Center. Paired-end sequences were generated on the Illumina MiSeq Personal Sequencer (2 x 150 nt read length). Reads in each pair for each sequencing run overlapped and paired ends were merged. The hamming distance (number of substitutions) was calculated for sliding overlaps of the two reads in a pair to find the best overlap (lowest hamming distance with a minimal overlap of 25 nucleotides and 98% identity). Merged sequences were binned according to barcode sequence and both barcode and amplicon primer sequences were trimmed using custom Perl scripts.

Sequence processing and analysis

Sequence data was processed and analyzed using the mothur program (Schloss et al., 2009). To ensure high quality data for analysis, sequence reads containing: ambiguous bases; homopolymers >7 bp; more than one mismatch in the primer sequence; or an average per base quality score below 35 within each 50 bp window were removed. Sequences that only appeared once in the total set were assumed to be a result of sequencing error and removed from the analysis. Chimeric sequences were removed from the data set using the UCHIME algorithm within the mothur program (Edgar et al., 2011). A random subset of 22,353 sequences per sample were used to balance read numbers and clustered into operational taxonomic units (OTUs). Taxonomy was assigned at a cutoff value of >90% (Hamilton et al., 2013). Using a 16S rRNA database prepared from the Ribosomal Database Project (RDP) 9, using the Bayesian method with a bootstrap algorithm (100 iterations) and a probability cutoff of 0.60 (Cole et al., 2009). Samples were clustered using the Fast UniFrac algorithm to generate trees and principle coordinate analysis (PCoA) plots (Hamady et al., 2010). The UniFrac algorithm was run using the Fast Unifrac program available at www.bmf2.colorado.edu/fastunifrac/.

Reagents

All solvents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise. LC-MS grade acetonitrile was purchased from Fisher Scientific (Pittsburgh, PA). Cholic acid, cholesterol, and diethyl phosphorocyanidate (DEPC) were purchased from Alfa Aesar (Ward Hill, MA). Isodeoxycholic acid was purchased from Steraloids (Newport, RI).

Liquid chromatography-mass spectrometry (LC-MS) analysis of fecal extracts

Fecal samples were suspended in 1 ml of 50% acetonitrile (w/v), and extracted by vortexing and sonication for 10 min. The suspension was centrifuged twice at 18,000 × *g* for 10 min, and after passing the supernatant through a 2 µm filter, the filtrate was transferred into a sample vial and subjected to LC-MS analysis. A 5 µl aliquot of fecal extract was injected into an Acquity™ UPLC system (Waters, Milford, MA) and separated by a mobile phase gradient ranging from H_2O to 95% aqueous acetonitrile containing 0.1% formic acid over a 10-min run. The LC eluant was introduced into a SYNAPT QTOF mass spectrometer (Waters) for accurate mass measurement and ion counting. Capillary and cone voltage for electrospray ionization (ESI) were maintained at -3 kV and -35 V for negative-mode detection, respectively. Source temperature and desolvation temperature were 120 °C and 350 °C, respectively. Nitrogen was used as a cone gas (50 l/h) and desolvation gas (600 l/h), and argon as the collision gas. For accurate mass measurement, the mass spectrometer was calibrated with sodium formate solution (range *m/z* 50-1000) and monitored by the intermittent injection of the lock mass leucine enkephalin ($[M-H]^-$ = 554.2615 *m/z*) in real time. Mass chromatograms and mass spectral data were acquired and processed by MassLynx™ software (Waters) in centroided format. Additional structural information was obtained via tandem MS (MS/MS) fragmentation with collision energies ranging from 15 to 30 eV. The concentration of bile acids in fecal samples was determined based on the peak areas of individual bile acids and external standards.

Chemometric analysis and biomarker identification

Chromatographic and mass spectral data of fecal samples were deconvoluted using MarkerLynx™ software (Waters). Each detected ion was represented by its retention time (RT) in the LC-system and its mass-to-charge ratio (*m/z*). A multivariate data matrix comprising sample identity, ion identity (RT and *m/z*) and relative ion abundance was generated through centroiding, deisotoping, filtering, peak recognition and integration, and was further exported into SIMCA-P+™ software (Umetrics, Kinnelon, NJ) (Chen et al., 2007). Unsupervised principal component analysis (PCA) and supervised orthogonal partial least squares (OPLS) analysis were used to analyze data from fecal extracts. Major latent variables in the data matrix were described in a scores scatter plot of established multivariate model. Fecal metabolites affected by FMT were identified by analyzing ions contributing to the separation of pre-FMT and post-FMT samples in the multivariate models (Chen et al., 2007; Bylesjo et al., 2008). The chemical identities of metabolites of interest were determined by accurate mass measurement, elemental composition analysis, database search (Lipid Maps: http://www.lipidmaps.org/, Human Metabolome Database: http://www.hmdb.ca/), MS/MS fragmentation, and comparisons with authentic standards.

Statistics

Experimental values for fecal metabolites are expressed as mean ± standard deviation (SD). Statistical analysis was performed using ANOVA followed by Tukey's HSD on significant groups at $\alpha = 0.05$. A *p*-value of <0.05 was considered statistically significant.

RESULTS

Complete patient recovery following one or two FMT procedures

Eleven of the 12 patients (91.6%) treated with FMT and analyzed in this study achieved clinical recovery with no recurrence of CDI following a single FMT over at least a one-year period of follow-up. Interestingly, while the patients were maintained on vancomycin prior to FMT, they continued to experience mild diarrheal symptoms (4.1 ± 1.3 stools per day; median type 5 on the Bristol stool scale). After the FMT procedure the frequency of stools decreased (2.0 ± 1.4 stools per day; median type 4 on the Bristol stool scale). Following a second FMT, the single patient with an initial recurrence of the disease also recovered. He was documented to be *C. difficile* toxin B negative and remained free of diarrhea for 6 months, at which point he experienced a relapse of CDI. This relapse was associated with a course of laxatives taken for constipation associated with his Parkinson's disease. The infection was successfully treated with a single 10-day course of fidaxomicin. Two patients were incidentally discovered to have underlying inflammatory bowel disease at the time of their FMT; one had Crohn's terminal ileitis and the second had lymphocytic colitis (Table 3.1). Neither patient required additional pharmacological therapy.

Composition of fecal communities dramatically changes following FMT

The composition of fecal microbiota in 14 patients before and after FMT, as well as each donor sample, was examined by sequencing the V6 region of the 16S rRNA gene. A total of 13,873,260 sequences were generated and 8,175,859 sequences remained after

quality filtering, removal of chimeras, and preclustering. Sequences were subsampled to 22,535 to obtain the same number of reads for each sample, clustered into operational taxonomic units (OTUs) at 90% sequence identity as used previously (Hamilton et al., 2013), and classified using the Ribosomal Database Project (RDP) (Cole et al., 2009).

As we previously showed for a small set of patients (Hamilton et al., 2013), fecal bacterial communities changed dramatically following FMT. Initially, α-diversity (diversity within each sample) was examined using the Shannon diversity index, which indicated that both donor samples and post-FMT samples were significantly more diverse than pre-FMT samples (Fig. 3.1A). To examine β-diversity (diversity between samples) principal coordinate analysis (PCoA) was performed based on UniFrac distances (Fig. 3.1B). The PCoA demonstrated that while donor and post-FMT samples clustered together along principal coordinate 1 (PC1), pre-FMT sample communities were distinct.

These shifts in diversity following FMT were related to changes in the relative abundance of the bacterial phyla *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* (Fig. 3.1C). Donor samples, representative of a healthy fecal microbiome (Zoetendal et al., 2008; Arumugam et al., 2011), were dominated by *Bacteroidetes* and *Firmicutes* (together accounting for ~80-90% of OTUs in each sample), with a very low abundance of *Proteobacteria* (~2% or fewer OTUs per sample). In contrast, patient samples prior to FMT tended to be dominated by the *Proteobacteria*, comprising at least 30% of OTUs in 13 of 14 samples (92.8%), and over 50% in seven samples. Following FMT, microbiota in patient samples more closely resembled the donors' composition, with increased abundance of *Firmicutes* and *Bacteroidetes* and decreased abundance of *Proteobacteria*. Overall, the number of *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and unclassified

bacterial OTUs significantly increased following FMT ($p=0.044$, $p=0.0015$, $p=0.0016$, and $p<0.0001$, respectively), while *Proteobacteria* and *Verrucomicrobia* significantly decreased ($p<0.0001$ and $p=0.035$, respectively) (Table 3.3).

Dramatic differences in community composition were also seen at the family level (Fig. 3.1D). Together, the bacterial families *Lachnospiraceae*, *Bacteroidaceae*, and *Ruminococcaceae* comprised 50% or more of the OTUs in each donor sample. In contrast, these families were found in much lower abundance in the majority of pre-FMT patient samples, with the *Lachnospiraceae* at less than 2% abundance in 11 of 14 samples (78.6%), the *Bacteroidaceae* at less than 1% abundance in each sample, and the *Ruminococcaceae* at less than 1% abundance in 13 of 14 samples (92.8%). Instead, *Enterobacteriaceae*, *Veillonellaceae*, and *Verrucomicrobiaceae*, minor constituents of donor communities, together represented ≥50% of OTUs in 13 of 14 pre-FMT samples (92.8%). Following FMT, these patterns reversed in most patient samples, with increased *Lachnospiraceae*, *Bacteroidaceae*, and *Ruminococcaceae*, and decreased *Enterobacteriaceae*, *Veillonellaceae*, and *Verrucomicrobiaceae*. Bacterial families with significantly altered OTU abundance after FMT compared to pre-FMT samples are shown in Table 3.4.

Changes in fecal bile acid composition following FMT

To explore changes in fecal bile acid composition induced by FMT, fecal samples collected from 14 CDI patients, before and after the FMT procedure, and six fecal samples from two donors were analyzed via LC-MS, together. Microbial composition in 12 of these patient sets and 4 donor samples (1 from donor 1, 3 from donor 2) were

analyzed above (Fig. 3.1). The profile of primary and secondary bile acids in fecal extracts was defined by examining the extracted ion chromatograms of deprotonated bile acids in the pre-and post-FMT samples. The results showed that FMT induced dramatic changes in fecal bile acid composition (Fig. 3.2A). Two primary bile acids, cholic acid (I) and chenodoxycholic acid (II), were present in significant amount in most pre-FMT samples, but were absent or existed in low abundance in most post-FMT and donor samples (Fig. 3.2B and C). In contrast, three secondary bile acids, lithocholic acid (III), deoxycholic acid (IV), and isodeoxycholic acid (V), were only present in the post-FMT and donor samples but were absent in the pre-FMT samples (Fig. 3.2D-F).

Metabolomic analysis of FMT-induced metabolic changes in the fecal extracts of R-CDI patients

To examine overall metabolic changes following FMT, the same fecal samples analyzed for bile acid composition were also analyzed via LC-MS-based metabolomics. Unsupervised principal component analysis (PCA) of chromatographic and mass spectral data acquired from LC-MS analysis revealed a clear separation of pre-FMT samples from the donor and post-FMT samples in a multivariate model (Fig. 3.3A). These findings suggest that FMT transformed the fecal metabolome of CDI patients to that consistent with the donors. The fecal metabolites which differed among pre-FMT, post-FMT, and donor samples were revealed in an S-plot of the supervised OPLS model (Fig. 3.3B and Table 3.5). Besides confirming the primary and secondary bile acids (I-V) as the fecal metabolites that distinguish pre-FMT and post-FMT samples, bile salts, including taurine conjugates (VI and VII, Fig. 3.3C-D) and glycine conjugates of cholic acid and

chenodeoxycholic acid (VIII and IX, data not shown) were identified as major contributors to sample separation in the multivariate models. The presence of significant amount of bile salts in many pre-FMT samples suggested their incomplete hydrolysis in these patients.

DISCUSSION

The appearance of hypervirulent strains of *C. difficile* infection has led to an increased incidence of recurrent and severe forms of the disease refractory to antibiotic therapies alone (Merrigan et al., 2010; Surawicz and Alexander, 2011; He et al., 2013). FMT has emerged as a solution to this unmet clinical need, and its use is becoming increasingly widespread in clinical practice (Borody and Khoruts, 2011; Surawicz and Alexander, 2011; Surawicz et al., 2013). However, the mechanisms behind the success of this therapy still remain largely unknown. Previous work by our group and others has demonstrated dramatic changes in the fecal microbiota of patients undergoing FMT, with fecal microbial communities becoming more similar to that of the donor, including an increased abundance of *Bacteroidetes* and *Firmicutes*, a decreased abundance of *Proteobacteria*, and an increase in overall microbial diversity (Khoruts et al., 2010; Shahinas et al., 2012; Hamilton et al., 2013; van Nood et al., 2013). The results presented in this study indicate that these microbiome changes also hold true for a larger sample size of patients. Consistent with the previous reports, FMT resulted in an increase in OTUs corresponding to *Bacteroidetes* and *Firmicutes* and a decrease in the abundance of *Proteobacteria*. The PCoA analysis also indicated that these changes represent shifts in the fecal bacterial community towards the composition of the donor and a shift from a highly altered community dominated by *Proteobacteria* towards the community typically found in healthy individuals (Zoetendal et al., 2008; Arumugam et al., 2011). The dramatic dysbiosis documented in the pre-FMT samples is likely induced and perpetuated

by continuous antibiotic pressure, mainly vancomycin, that the patients were taking at the time of collection.

Considering that gut microbiota in the colon have multiple metabolic functions, targeted metabolite profiling and untargeted metabolomics were adopted to examine the metabolic events induced by FMT. The results showed that after FMT the overall metabolic composition of samples shifted to one similar to that of the donors, mimicking our findings for taxonomic microbial composition. The bile acids and primary bile salts were by far the dominant metabolites contributing to the separation of pre-FMT samples from post-FMT and donor samples revealed by the multivariate model on the fecal metabolomes, suggesting that the changes in bile acid composition account for much of the overall change in fecal metabolome in R-CDI patients treated with FMT. Strikingly, secondary bile acids were simply not detectable in any of the pre-FMT fecal samples. Because of the well-known functions of gut microbiota in the hydrolysis of bile salts (taurine and glycine conjugates) to bile acids and the biotransformation of primary bile acids into secondary bile acids in the colon (Mahony et al., 1977; Stellwag and Hylemon, 1978; Hirano et al., 1981; Takamine and Imamura, 1995; Hofmann and Hagey, 2008), this observation suggests that pre-FMT patients in this study were deficient of microbiota that were capable of metabolizing secondary bile salts and bile acids, possibly due to the depletion or decrease of bile acid-metabolizing microbiota after extensive antibiotic treatment (Hashimoto et al., 1996; Carman et al., 2005; Toda et al., 2009). Therefore, FMT likely rehabilitated bile acid metabolizing microbiota in these same patients. It is even plausible that normalization of bile acid metabolism contributed to resolution of mild diarrheal symptoms experienced by patients while they were being maintained on

vancomycin prior to FMT. Indeed, increased content of primary bile acids in stool is correlated with diarrheal symptoms, while increased content of secondary bile acids correlates with constipation (Shin et al., 2013).

Increased concentrations of primary bile acids and their conjugates in R-CDI patients are consistent with relentless relapses of the infection each time suppressive antibiotics are discontinued. Taurocholic acid, which existed in high abundance in many pre-FMT samples, is a potent germinant for *C. difficile*, transforming inert spores into toxin-producing, free-living vegetative cells (Sorg and Sonenshein, 2008). In fact, taurocholic acid has been used for decades as a key component of laboratory medium used to germinate *C. difficile* spores (Wilson, 1983). In contrast, lithocholic acid and other secondary bile acids, which were deficient in pre-FMT samples but abundant in post-FMT and donor samples, inhibit *C. difficile* germination and colony growth (Sorg and Sonenshein, 2010; Giel et al., 2010). These findings suggest that changes in fecal bile acid composition observed in FMT patients following the procedure may generate an environment unsuitable to *C. difficile* germination or growth. Prompt restoration of normal fecal bile acid composition following FMT fits this hypothesis, as clinical recurrence of CDI would otherwise peak during the second and third weeks after discontinuation of antibiotics.

The changes in fecal bile acid composition of FMT patients may be directly related to changes in fecal bacterial composition. Many of the bacteria known to have 7α-dehydroxylation activity, which transforms primary bile acids into secondary bile acids, are members of the *Lachnospiraceae* and *Ruminococcaceae* families (i.e. Clostridium clusters XIVa and IV), including the best-studied of these species, *Clostridium scindens*

(Stellwag and Hylemon, 1978; sHirano et al., 1981; Takamine and Imamura, 1995; Doerner et al., 1997; Kitahara et al., 2000; Ridlon et al., 2006). By increasing the relative abundance of members of these bacterial families, it is possible that FMT increases 7α-dehydroxylation activity, leading to increased secondary bile acids and decreased primary bile acids. This may directly inhibit germination and thus toxin production by *C. difficile*.

In conclusion, our results support a mechanistic model where FMT prevents recurrence of CDI by restoring normal bile acid composition in the colon (Fig. 3.4). In this model, antibiotics used to treat CDI also inhibit normal members of the microbiota that hydrolyze bile salts to bile acids and then convert primary bile acids to secondary bile acids. The lack of bile acid metabolism in antibiotics-treated R-CDI patients generates a local environment that promotes germination of *C. difficile* spores and growth of new vegetative bacteria that actively produce toxins once antibiotic treatment is completed. Restoration of normal colonic microbial ecology by FMT restores bile acid metabolism and normal bile acid composition in the colon producing an unfavorable environment for *C. difficile* spore germination allowing clinical recovery of R-CDI patients. Therefore, microbiological or pharmacological strategies to manipulate the intestinal bile acid composition may become effective approaches for the treatment of R-CDI.

Table 3.1 - Clinical characteristics of the patient cohort.

Patient Number	1	2	3	4	5	6
Age	68	66	60	29	24	59
Gender	F	F	F	F	F	M
Total Number of C. difficile Episodes Prior to FMT	6	3	5	7	9	4
Duration of R-CDI Prior to FMT (months)	6	3	6	8	12	4
Original Trigger Event	Sinusitis	Spinal Surgery	Diverticulitis	C-section	Pelvic Pain	Bronchitis
Trigger Antibiotic	Not documented	Cephalosporin	Ciprofloxacin Metronidazole	Cephalosporin	Metronidazole	Azithromycin Ciprofloxacin
Prior hospitalization for severe or complicated CDI	No	Yes	Yes	No	No	Yes
Metronidazole Courses	2	1	2	2	2	1
2-week Vancomycin Courses	1	1	1	3	4	1
Vancomycin Taper/Pulse	2	1	2	2	1	1
Fidaxomicin	1	0	0	0	2	1
Rifaximin Chaser	0	0	0	1	0	0
Intercurrent Antibiotics	None	None	None	None	None	None
Immunosuppression	None	None	None	None	None	None
Probiotics	Florastor	None	Florastor	None	Florastor	Lactobacillus rhamnosus
Findings at Colonoscopy	Normal Examination	Diverticulosis, severe	Diverticulosis, moderate	Normal Examination	Normal Examination	Diverticulosis, moderate
Type of Fecal Microbiota Preparation	Frozen	Frozen	Fresh	Frozen	Fresh	Fresh
Donor	1	1	1	1	2	1
Resolution of CDI	Yes	Yes	Yes	Yes	Yes	Yes

Patient Number	7	8	9	10	11	12
Age	56	87	65	52	47	52
Gender	F	F	F	F	F	F
Total Number of C. difficile Episodes Prior to FMT	5	8	5	8	4	9
Duration of R-CDI Prior to FMT (months)	6	9	7	11	4	7
Original Trigger Event	Knee Surgery	Duodenal Ulcer Perforation	Vaginal Infection	Urinary Tract Infection	Sinusitis	Pelvic Reconstruction Surgery
Trigger Antibiotic	Cephalosporin	Not documented; multiple antibiotics	Clindamycin	Ciprofloxacin	Not documented	Clindamycin
Prior hospitalization for severe or complicated CDI	No	Yes	No	No	Yes	No
Metronidazole Courses	1	2	2	2	1	1
2-week Vancomycin Courses	1	2	1	3	1	2
Vancomycin Taper/Pulse	1	1	2	2	1	4
Fidaxomicin	1	1	0	2	0	1
Rifaximin Chaser	1	1	0	0	1	1
Intercurrent Antibiotics	None	None	None	None	None	None
Immunosuppression	None	None	None	None	None	None
Probiotics	None	Florastor	None	None	None	Acidophilus Probiotic Blend
Findings at Colonoscopy	Terminal Ileitis (Crohn's)	Normal Examination	Normal Examination	Normal Examination	Lymphocytic Colitis	Diverticulosis, mild
Type of Fecal Microbiota Preparation	Frozen	Frozen	Fresh	Frozen	Fresh	Frozen
Donor	1	1	1	1	1	1
Resolution of CDI	Yes	Yes	Yes	Yes	Yes	Yes

Patient Number	13	14	15	16
Age	71	72	79	83
Gender	M	M	F	F
Total Number of C. difficile Episodes Prior to FMT	5	6	4	3
Duration of R-CDI Prior to FMT (months)	11	7	5	5
Original Trigger Event	Multiple Infections, including pneumonia	Diarrheal Illness of Unclear Cause	Bladder Infection	Urinary Sepsis
Trigger Antibiotic	Multiple antibiotics	No identified antibiotic trigger	Not documented	Not documented; multiple antibiotics
Prior hospitalization for severe or complicated CDI	Yes	Yes	Yes	No
Metronidazole Courses	2	2	0	1
2-week Vancomycin Courses	1	2	2	1
Vancomycin Taper/Pulse	3	2	2	1
Fidaxomicin	0	0	0	0
Rifaximin Chaser	0	0	0	0
Intercurrent Antibiotics	Ciprofloxacin	None	None	None
Immunosuppression	Tacrolimus; Mycophenolate Mofetil	None	None	None
Probiotics	Lactobacillus rhamnosus	None	None	None
Findings at Colonoscopy	Diverticulosis, mild	Diverticulosis, severe	Adenomatous polyps; moderate diverticulosis	Non-diagnostic inflammation in the lamina propria
Type of Fecal Microbiota Preparation	Fresh	Fresh	Frozen	Frozen
Donor	1	1	1	2
Resolution of CDI	Yes	No	Yes	Yes

R-CDI = Recurrent *C. difficile* infection; severe CDI = WBC ≥ 15,000 cells/mm^3, albumin < 3, abdominal tenderness or documentation of colitis on CT scan; complicated CDI = one of the following: ICU admission, WBC ≥ 35,000 cells/mm^3, albumin < 2.5, hypotension, fever > 38.5°C, mental status changes, evidence of end-organ failure; intercurrent antibiotics = antibiotics used to treat non-*C. difficile* infection after CDI diagnosis.

Table 3.2 - Primers for V6 16S PCR.

Primer Name	Sequence (5’ - 3’)
Forward Primer 1	[*]NCNACGCGAAGAACCTTANC
Forward Primer 2	[*]NNCAACGCGAAAAACCTTACC
Forward Primer 3	[*]NNNCAACGCGCAGAACCTTACC
Forward Primer 4	[*]NNNNATACGCGARGAACCTTACC
Forward Primer 5	[*]NNNNNCTAACCGANGAACCTYACC
Reverse Primer	[**][6 bp Index sequence][†][4-7 N] CGACRRCCATGCANCACCT

***** = Illumina TruSeq Universal Adapter sequence
****** = Illumina PCR primer
† = Illumina multiplexing PCR primer 2.0

Table 3.3 - Abundance of major bacterial phyla in patient and donor samples.

	Average no. OTUs (of 22,535 total)			
Phylum	**Donor**	**Pre-FMT**	**Post-FMT**	**p-value (pre vs. post-FMT)**
Actinobacteria	275	24	219	0.044
Bacteroidetes	8316	36	4402	0.0015
Firmicutes	10,223	5412	12,443	0.0016
Fusobacteria	0	480	2	0.177
Proteobacteria	207	12,689	1234	<0.0001
Verrucomicrobia	189	3469	449	0.035
Bacteria-unclassified	3302	424	3785	<0.0001

Table 3.4 - Significantly changed bacterial families in patient and donor samples.

	Average no. OTUs (of 22,535 total)			
Family	**Donor**	**Pre-FMT**	**Post-FMT**	**p-value (pre vs. post-FMT)**
Bacteroidaceae	6451	25	4348	0.0009
Coriobacteriaceae	150	12	150	0.034
Enterobacteriaceae	13	11,572	1083	<0.0001
Lachnospiraceae	4002	286	4737	<0.0001
Lactobacillaceae	2	255	12	0.0019
Rikenellaceae	388	2	455	0.0014
Ruminococcaceae	1749	112	3797	0.0002
Veillonellaceae	117	1261	81	0.0006
Verrucomicrobiaceae	189	3469	449	0.035
Bacteroidetes-unclassified	2185	501	1809	0.0064

Table 3.5 - Fecal metabolite markers of FMT.

ID	$[M-H]^-$	Formula	Identity	Effect of FMT
I	407.279	$C_{24}H_{40}O_5$	cholic acid (CA)	↓
II	391.284	$C_{24}H_{40}O_4$	chenodeoxycholic acid (CDCA)	↓
III	391.284	$C_{24}H_{40}O_4$	deoxycholic acid (DCA)	↑
IV	375.289	$C_{24}H_{40}O_3$	lithocholic acid (LCA)	↑
V	391.284	$C_{24}H_{40}O_4$	isodeoxycholic acid (isoDCA)	↑
VI	514.283	$C_{26}H_{45}NO_7S4$	taurocholic acid (TCA)	↓
VI	498.288	$C_{26}H_{45}NO_6S$	taurochenodeoxycholic acid (TCDCA)	↓
VI	464.281	$C_{26}H_{43}NO_6$	glycocholic acid (GCA)	↓
IX	448.306	$C_{26}H_{43}NO_5$	glycoochenodeoxycholic acid (GCDCA)	↓

Major primary and secondary bile acids and the conjugates of primary bile acids (I-IX) were identified as the metabolite markers contributing to the separation of the pre-FMT samples from the post-FMT and donor samples in the metabolomic analysis. $[M-H]^-$ = mass-to-charge ratio of deprotonated metabolite; ↑ = increased after FMT; ↓ = decreased after FMT.

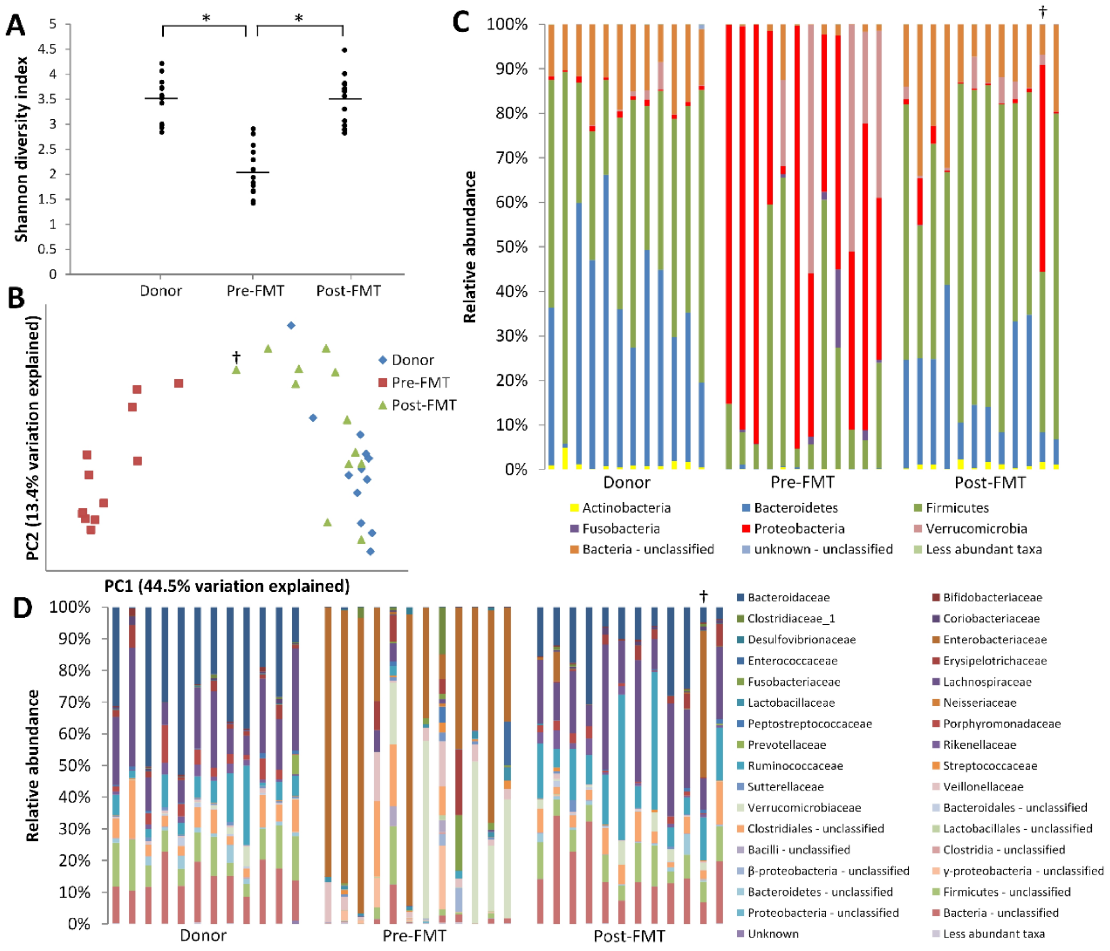


Figure 3.1 - Fecal bacterial communities change following FMT. A) Shannon diversity indices of fecal samples. Legend: * = p <0.05. B) Principal coordinate analysis of UniFrac distances between bacterial communities. PC1 = Principal coordinate 1, PC2 = Principal coordinate 2. C) Relative abundance of OTUs from bacterial phyla in fecal samples. Colors correspond to phyla (see key below figure). D) Relative abundance of OTUs from bacterial families in fecal samples. Colors correspond to families (see key to right of figure). Legend: † = patient who failed initial FMT.

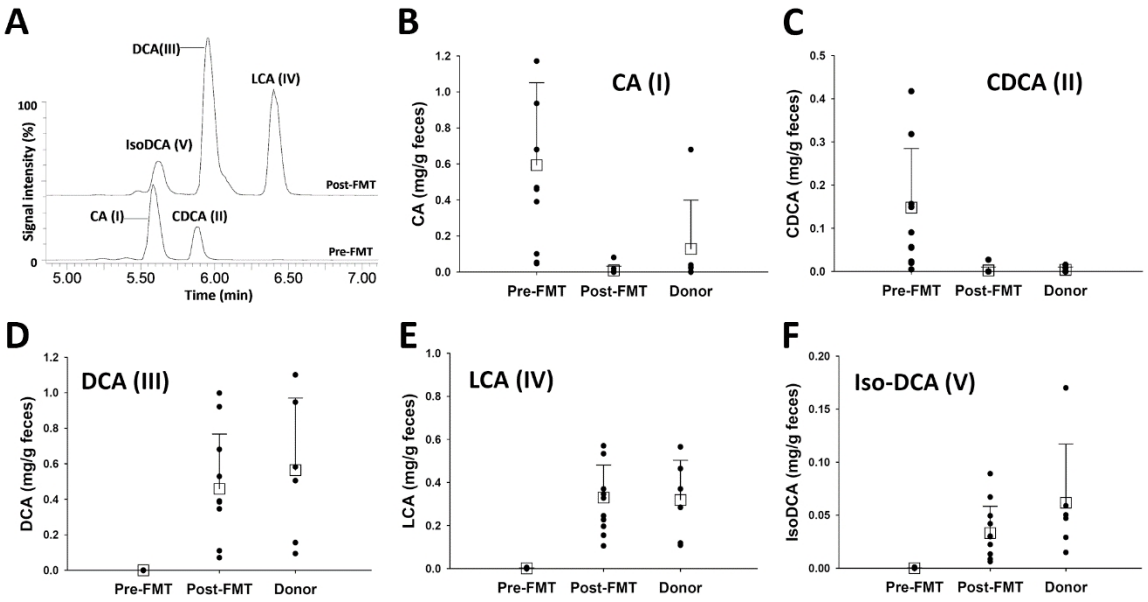


Figure 3.2 - Bile acid concentrations are significantly altered after FMT. Conditions for LC-MS analysis are described in the *Methods*. A) The overlay of representative chromatograms of bile acid metabolites in pre-FMT and post-FMT fecal extracts. The chromatograms were generated by extracting MS signals within 20 ppm of calculated exact masses (407.2797, 391.2848, and 375.2899 *m/z* in negative mode) of primary and secondary bile acids of sterol metabolites (I - V). The signal intensity of deoxycholic acid (III) in the post-FMT sample was arbitrarily set as 100%. B-F) Concentration of bile acids (I - V) in the pre-FMT, post-FMT, and donor samples. Legend: * = p <0.05.

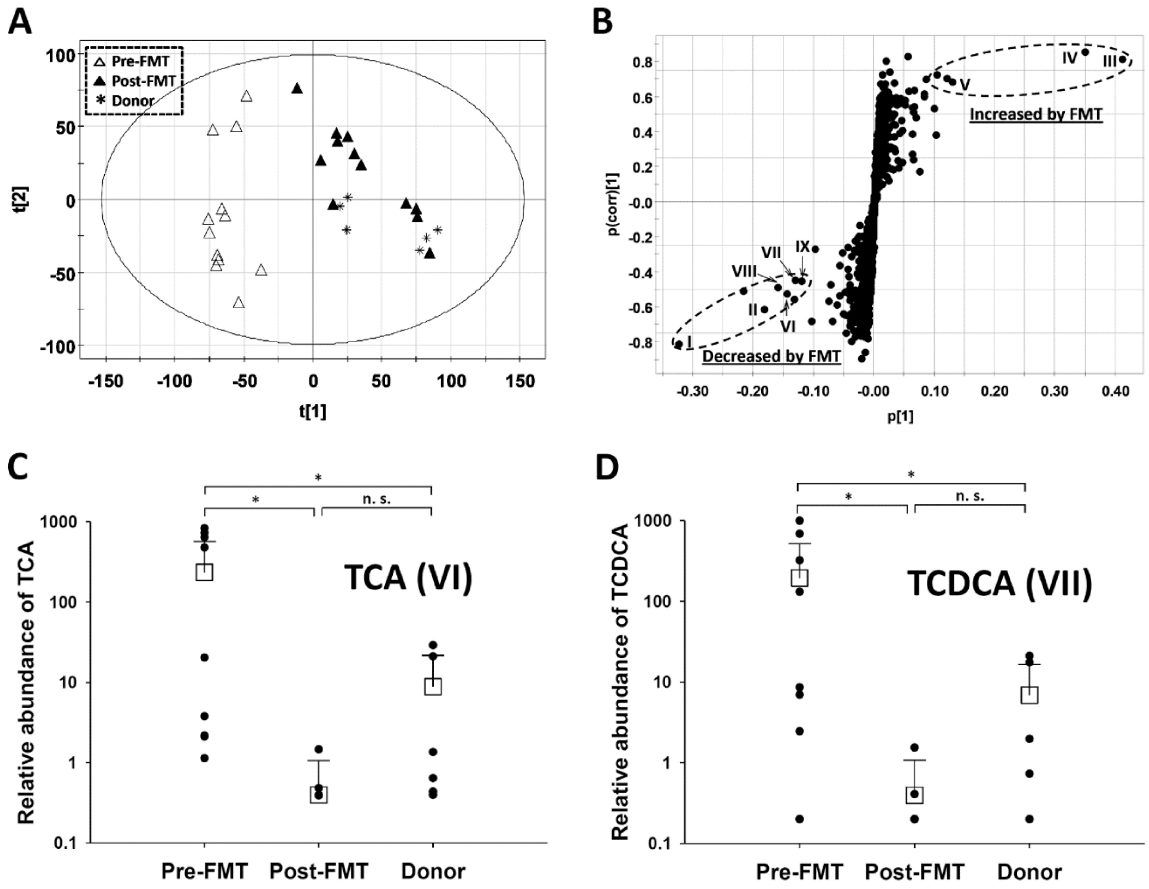


Figure 3.3 - Identification and characterization of FMT-responsive sterol metabolites in fecal extracts of CDI patients. Multivariate analysis of LC-MS data from fecal extracts is described in the *Methods*. A) The scores plot of a PCA model on pre-FMT (▲), post-FMT (Δ), and donor (*) samples. The *t*[1] and *t*[2] values represent the scores of each sample in the principal component 1 and 2, respectively. B) The loadings plot of PCA model. Sterol metabolites contributing to the separation of pre-FMT and post-FMT samples (I - IX) were labeled and their chemical identities are listed in Table 3. C-D) Relative abundances of taurine conjugates of primary bile acids (VI and VII) in the pre-FMT, post-FMT, and donor samples. Legend: * = $p < 0.05$.

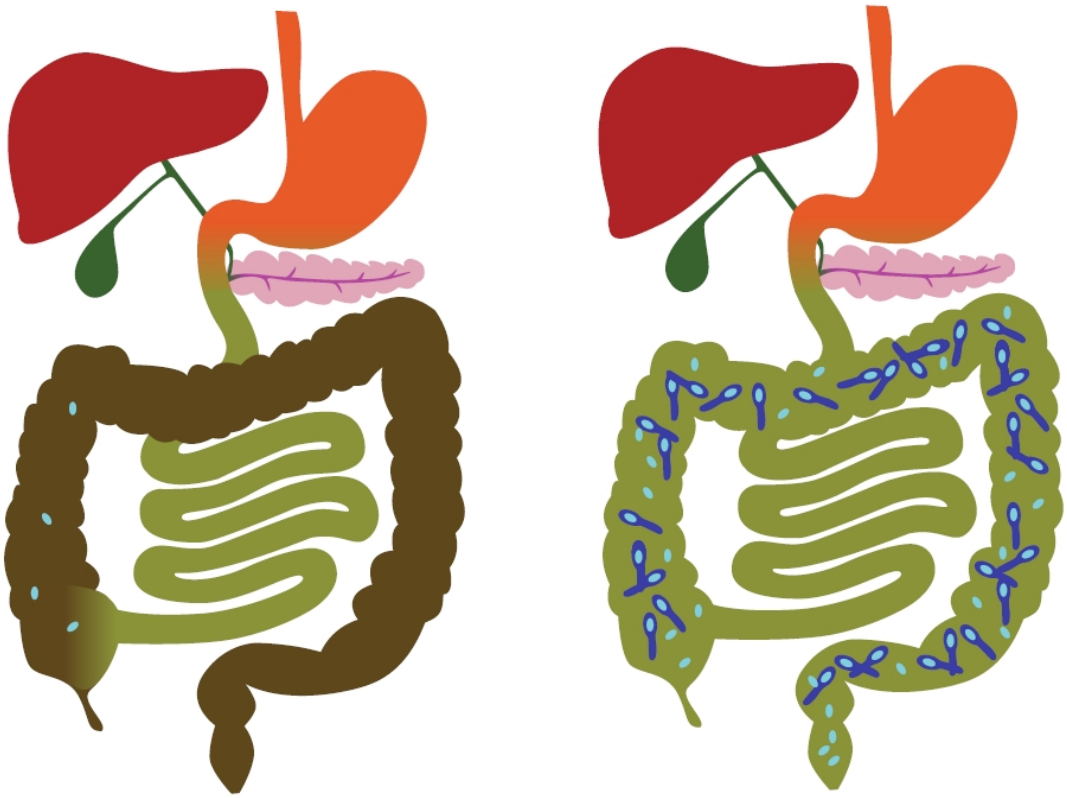


Figure 3.4 - Antibiotics promote recurrence of *C. difficile* infection by decreasing hydrolysis of bile salts and conversion of primary bile acids into secondary bile acids (the model). A) Normal bile acid composition in the colon (shown in brown color) prevents germination of *C. difficile* spores. B) Antibiotics allow increased levels of bile salts and primary bile acids in the colon (shown in green color), which promote germination of *C. difficile* spores and growth of vegetative forms of bacteria.

Chapter 4

Fecal Bile Acid Composition is a Mechanism of Fecal Microbiota Transplantation Which Controls *Clostridium difficile* Germination and Growth

SUMMARY

Fecal microbiota transplantation (FMT) is a highly effective therapy for recurrent *Clostridium difficile* infection (R-CDI), but its mechanisms remain poorly understood. Emerging evidence suggests that bile acids in the gut have significant effects on *C. difficile* physiology, and therefore on patient susceptibility to recurrent infection. We analyzed germination of spores from 10 clinical *C. difficile* isolates exposed to combinations of bile acids present in patient feces before and after FMT. Bile acids present in feces prior to FMT induced germination of *C. difficile*, while those present after FMT did not. Furthermore, post-FMT bile acids inhibited vegetative growth of *C. difficile*. Ursodeoxycholic acid (UDCA), a minor human secondary bile acid clinically available as a therapeutic, also inhibited *C. difficile* germination and growth. Finally, we sequenced the newly-identified germinant receptor in *C. difficile*, CspC, and found that variations in germination response across isolates corresponded to mutations in this receptor. These results indicate that changes to fecal bile acids are in fact a key mechanism of FMT, that differences in the germinant receptor correspond to differences in response to these bile acids, and that bile acids can be developed into effective non-antibiotic pharmacologic agents which may treat R-CDI.

INTRODUCTION

Clostridium difficile infection (CDI) has become one of the most common nosocomial infections in developed countries in the last 20 years, and more recently has become an important cause of community-acquired infectious colitis (Kelly and Lamont, 2008; Miller et al., 2011; Ananthrakishnan, 2011; Lessa et al., 2012; Khanna et al., 2012; Chitnis et al., 2013). Unfortunately, antibiotic therapy alone commonly fails to cure this disease. In fact, the use of antibiotics is the primary risk factor for CDI and perpetuates its recurrence (Kelly and Lamont, 2008; Miller et al., 2011; Ananthrakishnan, 2011; Lessa et al., 2012; Khanna et al., 2012; Chitnis et al., 2013; Borody and Khoruts, 2011; Hu et al., 2009). Approximately 20-30% of patients experience recurrence of CDI after the initial infection, and the risk of recurrence increases with each subsequent antibiotic treatment (Kelly and Lamont, 2008; Borody and Khoruts, 2011; Louie et al., 2011; Surawicz and Alexander, 2011). After three or more relapses, patients typically develop recurrent CDI syndrome (R-CDI), a state of persistent infection that is resistant to existing antibiotic regimens (Borody and Khoruts, 2011; Surawicz and Alexander, 2011). However, fecal microbiota transplantation (FMT), in which fecal material from a healthy donor is delivered into the gastrointestinal tract of a patient, is increasingly employed in clinical practice as a highly effective method to break the cycle of R-CDI (Gough et al., 2011; Hamilton et al., 2012; van Nood et al., 2013; Youngster et al., 2014; Youngster et al., 2015).

Despite the high clinical efficacy of FMT, its mechanisms are poorly understood. It is known that the fecal microbiota of R-CDI patients undergoes compositional

normalization following the procedure (Khoruts et al., 2010; Shahinas et al., 2012; Hamilton et al., 2013; van Nood et al., 2013; Seekatz et al., 2014; Fuentes et al., 2014; Shankar et al., 2014). This normalization is associated with functional restoration of fecal bacterial bile acid metabolism in these patients, including restoration of bacterially-mediated conversion of primary to secondary bile acids (Weingarden et al., 2014). Some primary bile acids, such as taurocholate (TA), are potent germinants for *C. difficile* spores, while certain secondary bile acids act as inhibitors of both germination and vegetative growth of the bacterium (Sorg and Sonenshein, 2008; Sorg and Sonenshein, 2009; Sorg and Sonenshein, 2010; Giel et al., 2010; Heeg et al., 2012), though no work has yet examined the effects of a combination of bile acids which reflects the colonic environment in humans with or without CDI. Based on these observations, we hypothesized that antibiotics used to treat CDI patients alter bacterially-mediated bile acid metabolism towards a composition that favors *C. difficile* germination, and that FMT prevents *C. difficile* expansion via restoration of gut microbial ecology and normalization of colonic bile acid composition.

In this report, we demonstrate that the combination of bile acids present in the feces of R-CDI patients prior to FMT induced germination of multiple clinical isolates of *C. difficile*, while the combination of bile acids present after FMT did not. We also report that ursodeoxycholic acid (UDCA), a minor secondary bile acid in humans that is currently approved for certain cholestatic conditions (Poupon, 2010; Lindor et al., 2009), inhibits *C. difficile* germination and growth *in vitro*. Finally, we show that variations in germination response across different strains may be related to variation in the *C. difficile* germinant receptor. These findings support the hypothesis that restoration of secondary

bile acid metabolism is an important mechanism of FMT, and that novel pharmacological interventions based on this insight can be developed to treat R-CDI.

MATERIALS AND METHODS

Isolation and characterization of C. difficile isolates

Isolation of *C. difficile* from environmental samples was done using a protocol developed by the CDC and modified for the present study. Sterile phosphate buffered saline, pH 7, with 0.1% Tween 80 (50 mL) was added to sterile bags containing environmental sample sponges. Bags were placed into a Stomacher 400 circulator (Seward Laboratory Systems, Davie, FL) and macerated at 260 RPM for 1 min. The liquid was removed and centrifuged at 3500x*g* for 15 min. The pellet was resuspended in the remaining buffer and a 0.2 mL aliquot of the resulting suspension was plated, in duplicate, onto pre-reduced cycloserine-cefoxitin-fructose agar with horse blood and taurocholate (CCFA-HT, Anaerobic systems, USA). 1 mL of suspension was inoculated into cycloserine-cefoxitin-fructose broth (CCFB) (Arroyo et al., 2005). CCFA-HT plates and CCFB tubes were incubated for 48-72 hr at 37ºC under anaerobic conditions. *C. difficile* colonies from CCFA-HT plates were identified using McLung Toabe agar (lecithinase and lipase-negative), blood agar (no hemolysis), PRO kit (Remel, USA), and Gram staining (Gram-positive spore forming bacilli). Presumptive *C. difficile* colonies were further characterized by PCR detection of the pathogenicity locus (PaLoc), binary toxin (*cdtB*), and *C. difficile* toxin regulator *tcdC* genes; toxinotyping; and sequence analysis of the *tcdC* gene for specific base pair deletions (Braun et al., 1996; Rupnik et al., 1998; Stubbs et al., 2000; Spigaglia and Mastrantonio, 2002). Confirmed *C. difficile* isolates also underwent pulsed-field gel electrophoresis (PFGE) analysis, allowing assignment to an established or novel North American pulsotype (NAP) based on an 80% similarity

threshold in comparison with CDC reference profiles (Killgore et al., 2008). Confirmed isolates were stored in 25% glycerol at -80°C.

C. difficile spore isolation

C. difficile cells from frozen stocks were inoculated into CCFB medium and grown anaerobically at 37°C for 48 hr. Cultures were plated onto brain heart infusion with 5 g/L yeast extract and 0.1% L-cysteine (BHIS) and grown for 7 d at 37°C under anaerobic conditions. Following Sorg and Sonenshein, 2010, colonies from each plate were scraped into 1 mL of ice-cold water and incubated at 4°C overnight to release spores. A 3 mL suspension was loaded onto 10 mL of 50% (w/v) sucrose in a 15 mL conical tube and centrifuged in a swinging bucket rotor at 3200 x *g* for 20 min at 4°C. Sucrose and vegetative cells above the spore pellet were removed, and the pellet was washed 5 times in ice-cold water to remove remaining sucrose. Spores were examined under phase-contrast microscopy to determine purity; spore samples with >99% purity (<1% vegetative cells) were stored at 4°C.

C. difficile spore germination

Germination assays were done as previously described by Sorg and Sonenshein, 2010. Spores were heated to 65°C for 30 min and inoculated into BHIS, with or without bile acids, within an anaerobic bag flushed and filled with N_2 gas. OD_{600} was measured initially ($OD6_{00}(t_0)$) and every minute for 20 min ($OD_{600}(t)$) using an EL808 Microplate Reader (Biotek Instruments, Inc., Winooski, VT). Relative OD_{600} for each time point was calculated as $OD_{600}(t)/OD_{600}(t_0)$. Experiments were performed in triplicate.

Growth of C. difficile vegetative cells

Cells from frozen stocks were inoculated into BHIS broth with 0.1% (w/v) taurocholate and incubated overnight at 37°C under anaerobic conditions. Vegetative cells were inoculated into tubes containing BHIS, with or without bile acids, normalized to an OD_{600} of 0.005, and grown anaerobically at 37°C. Measurements of OD_{600} were collected each hour for 12 hr following inoculation, and a final OD_{600} was measured after 24 hr growth. Experiments were performed in triplicate.

Sequencing of C. difficile germinant receptor

Two sets of PCR primers were designed based on a published DNA sequence of the *C. difficile* germinant receptor CspC (Francis et al., 2013) (Table 4.4). For each primer set, three PCR reactions were run in order to generate ~700 bp overlapping fragments for Sanger sequencing. Samples were amplified in 50 µL reactions containing 1x PCR buffer with 1.5 mM magnesium chloride, 0.2 µM nucleotides, 0.4 µM one forward and reverse primer, 2.5 U Choice Taq (Denville Scientific, South Plainfield, NJ), and 25 ng of template. Reactions were heated to 95°C for 3 min, then subjected to 25 cycles of 95°C for 30 sec, 51°C (forward primers 1A, 1B, and 1C), 57°C (primer 2B), or 58°C (primers 2A and 2C) for 30 sec, and 72°C for 150 (primers 1A and 2A), 90 (primers 1B and 2B), or 60 (primers 1C and 2C) sec, before a final elongation step at 72°C for 15 min. Amplicons were purified with the QIAquick PCR purification kit (Qiagen, Germantown, MD) and sequenced via Sanger sequencing. Sequences were manually edited using Chromas Lite (Technelysium Pty Ltd, Australia) and assembled into full-length *cspC*

sequences, then translated into protein sequences using the ExPASy protein translate tool (Gasteiger et al., 2003). Protein sequences were aligned and UPGMA tree (with boot-strapping) was generated using Clustal X (Larkin et al., 2007).

Reagents

Sodium taurocholate, sodium cholate, sodium deoxycholate, chenodeoxycholic acid (CDCA), lithocholic acid (LCA), and ursodeoxycholic acid (UDCA) were obtained from Fisher Scientific (Waltham, MA). The CDCA, LCA, and UDCA were dissolved in 100% ethanol prior to use.

Statistical analysis

Analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD) test was used to determine statistical significance of germination and growth assay results.

Study approval

The study was approved by the University of Minnesota Institutional Review Board (IRB). Collection of household *C. difficile* isolates was performed under IRB 1112M07323. The study of fecal specimens was performed under IRB 0901M56962. All human subjects provided informed consent for participation in the study.

RESULTS

Fecal bile acids present before FMT induce germination of C. difficile spores

An established spectrophotometric assay that relies on the refractive nature of bacterial spore coats was used to examine germination of *C. difficile* (Sorg and Sonenshein, 2008; Sorg and Sonenshein, 2009; Sorg and Sonenshein, 2010; Heeg et al., 2012). Spore coats are refractive towards light and appear phase-bright under phase-contrast microscopy, but are rapidly degraded during the initial step of germination and become phase-dark (Moir and Smith, 1990) (Fig. 4.1A). This degradation can be measured as a decrease in light absorption at 600nm (OD_{600}), allowing for the measurement of germination of *C. difficile* spores independent of subsequent growth of the resulting vegetative cells. This decrease in OD_{600} is measured as a relative OD_{600}, or OD_{600} at a given time normalized to initial OD_{600}. We validated this assay by correlating relative OD_{600} to the appearance of phase-dark spores and vegetative cells via phase-contrast microscopy and to the percent of colony-forming units (CFUs) which resulted from plating these cells on BHIS (Fig. 4.1A). A decrease of 16.5% of initial OD_{600} (0.165) after 20 min exposure to 2 mM of the germinant TA in BHIS corresponded to an increase of 69.1% phase-dark and vegetative cells compared to spores exposed to BHIS alone. In addition, exposure to 2 mM TA resulted in colony formation by 0.1% of total cells, compared to no detectable growth from cells exposed to BHIS alone. Having demonstrated that a decrease in OD_{600} corresponds to spore coat loss and colony outgrowth, we used this germination assay to determine whether bile acids present in patient feces before or after FMT induce germination of *C. difficile* spores.

We previously demonstrated that treatment of R-CDI patients with FMT results in conversion of fecal bile acid composition from domination by primary bile acids to domination by secondary bile acids (Weingarden et al., 2014). Here we exposed *C. difficile* spores from a North American Pulsed-field gel electrophoresis type 1 (NAP1) strain, which is typically considered hypervirulent (McDonald et al., 2005), to combinations of bile acids at concentrations present in patient feces before FMT (pre-FMT bile acids) and after FMT (post-FMT bile acids) (Table 4.1 and Fig. 4.1B). The relative OD_{600} of spores exposed to pre-FMT bile acids significantly decreased ($p<0.05$) compared to spores exposed to post-FMT bile acids, which suggests that the combination of intracolonic bile acids prior to FMT induce germination of *C. difficile* spores, while the combination of bile acids present after FMT do not.

Although these findings indicated that a single isolate of *C. difficile* germinates in response to pre-FMT bile acids combinations, previous work has shown that the germination response to bile acids can vary among different strains of *C. difficile* (Heeg et al., 2012). To test whether the germination response to fecal bile acid combinations varied across *C. difficile* strains, we tested spores from nine additional *C. difficile* isolates, along with the NAP1 strain above for a total of 10 isolates, which were collected from the household environment of R-CDI patients treated with FMT in our program. These 10 isolates included representatives from four different NAP types and three distinct toxinotypes (Table 4.2). When spores from all 10 isolates were exposed to pre-FMT or post-FMT bile acid combinations, there was a significant ($p<0.05$) decrease in the relative OD_{600} of spores exposed to pre-FMT bile acids versus post-FMT (Fig. 4.1C, Table 4.3). These results indicate that the typical fecal bile acid composition before FMT

can induce germination in multiple strains of *C. difficile*, whereas bile acids present after FMT do not.

To investigate how much each bile acid in the pre-FMT combination individually contributed to *C. difficile* germination, we examined the effects of a range of concentrations of primary bile acids on the relative OD_{600} of spores. Taurocholate, which has long been known as a germinant for *C. difficile* spores (Wilson, 1983; Sorg and Sonenshein, 2008) caused a significant ($p<0.001$) decrease in relative OD_{600} of NAP1 spores alone and of spores from all 10 isolates (Fig. 4.2A, Table 4.3). Cholate has also been considered a germinant for *C. difficile* (Wilson, 1983; Sorg and Sonenshein, 2008). While we found no significant decrease in relative OD_{600} after cholate exposure for spores from the NAP1 isolate alone, the relative OD_{600} of spores from all 10 isolates decreased significantly following exposure to 1 and 2 mM cholate ($p<0.05$ and $p<0.01$, respectively) (Fig. 4.2B). These findings suggest that both taurocholate and cholate induce germination in spores from these clinical isolates of *C. difficile*.

In contrast to taurocholate and cholate, chenodeoxycholic acid (CDCA), another major primary bile acid in our pre-FMT combination, is typically found to inhibit *C. difficile* germination (Sorg and Sonenshein, 2009; Sorg and Sonenshein, 2010). Consistent with this, we found that exposure to CDCA significantly ($p<0.01$) inhibited the decrease in relative OD_{600} seen with taurocholate alone for spores from both the NAP1 isolate alone and spores from all 10 isolates (Fig. 4.3). This result indicates that CDCA inhibits germination of spores from our *C. difficile* isolates. While exposure to CDCA without taurocholate did not decrease the relative OD_{600} of NAP1 spores alone, 2 mM CDCA caused a significant ($p<0.05$) decrease in relative OD_{600} for all 10 isolates

(Fig. 4.2C, Table 4.3). This suggests that higher concentrations of CDCA can induce germination in some isolates of *C. difficile*. Overall, these findings indicate that all three primary bile acids that predominate in pre-FMT feces (taurocholate, cholate, and CDCA) can induce germination of *C. difficile* at sufficiently high concentrations. Furthermore, although CDCA can also inhibit taurocholate-mediated germination, the effects of cholate and taurocholate outweigh the effects of CDCA at the concentrations present in pre-FMT patient feces (Table 4.1).

We next investigated the individual effects of bile acids in the post-FMT combination on *C. difficile* germination. Deoxycholate, the most abundant bile acid in the post-FMT combination, has been shown to cause germination of *C. difficile* (Sorg and Sonenshein, 2008). We tested whether physiological concentrations of deoxycholate led to germination of our isolates by exposing spores to a range of concentrations inclusive of the average concentration of this bile acid in post-FMT patient feces. There was no decrease in relative OD_{600} of spores from the NAP1 isolate alone or of spores from all 10 isolates after exposure to any tested concentration of deoxycholate (Fig. 4.4A, Table 4.3). Similarly, no decrease in relative OD_{600} was observed when spores were exposed to any tested concentration of lithocholic acid, the other secondary bile acid present in the post-FMT bile acid combination (Fig. 4.4B, Table 4.3). These results indicate that the secondary bile acids deoxycholate and lithocholic acid, which are elevated in post-FMT patient feces, do not induce germination of *C. difficile* spores.

Overall, our findings demonstrate that primary bile acids at the concentrations observed in patient feces prior to FMT can induce *C. difficile* spores to germinate, likely due to the dominant effects of cholate and taurocholate. Furthermore, the secondary bile

acids deoxycholate and lithocholic acid do not induce germination of *C. difficile* at the concentrations found in patient feces after FMT.

Fecal bile acids present after FMT strongly inhibit vegetative growth of C. difficile

We also investigated whether pre- and post-FMT bile acid combinations affect vegetative growth of *C. difficile*. Overnight cultures of all 10 isolates were grown with taurocholate to induce germination, and resulting vegetative cells were inoculated (to OD_{600} = 0.005) into BHIS medium or BHIS containing pre-FMT or post-FMT bile acid combinations. The growth of cells in BHIS containing either pre-FMT bile acids or post-FMT bile acids was delayed relative to cells grown in BHIS alone (Fig. 4.5A, Fig. 4.6). However, in 9 out of 10 isolates, the OD_{600} of cells after 24 h in medium with post-FMT bile acids was significantly ($p<0.05$) lower than cells grown with pre-FMT bile acids. This suggests that secondary bile acids present in post-FMT patient feces also inhibit vegetative growth of *C. difficile* compared to bile acids found in patient feces before FMT (Fig. 4.5B, Fig. 4.6). This effect was not likely due to a decrease in pH from the addition of bile acids, since the pH of BHIS alone was not significantly different from the pH of BHIS containing either combination of bile acids (data not shown).

UDCA inhibits germination and vegetative growth of C. difficile

Our findings confirmed that secondary bile acids present in patient feces following FMT do not induce germination and significantly inhibit growth of vegetative *C. difficile*. We therefore hypothesized that the one secondary bile acid currently

approved for use in human patients, UDCA, could also inhibit *C. difficile* germination and growth, and that UDCA might therefore be a novel treatment for CDI.

To explore UDCA as a therapeutic agent against *C. difficile*, we tested whether UDCA could inhibit taurocholate-mediated germination of our 10 isolates (Table 4.2). Compared to spores exposed to taurocholate alone, addition of any concentration of UDCA significantly ($p<0.01$) blunted the decrease in relative OD_{600} of spores from these 10 isolates (Fig. 4.7, Table 4.3). These findings indicate that UDCA can prevent taurocholate-induced germination of multiple *C. difficile* isolates.

Having demonstrated that UDCA can significantly inhibit germination of *C. difficile*, we examined whether the drug could also affect vegetative growth. As above, overnight cultures of vegetative cells from each isolate were inoculated into tubes containing BHIS or BHIS with 2 mM UDCA. Isolates grown with UDCA showed substantially delayed growth compared to control cultures, and after 24 h the OD_{600} of cultures grown with UDCA was significantly ($p<0.001$) lower than controls (Fig. 4.8, Fig. 4.9). These findings suggest that UDCA inhibits growth of vegetative *C. difficile* in addition to inhibiting spore germination.

Variation in germination response to UDCA may be explained by variations in the C. difficile germinant receptor

Although our findings suggested that pre-FMT bile acids induce *C. difficile* spore germination across a range of strains, previous work has indicated that the response of spores from different isolates and strains of *C. difficile* to bile acids may vary (Heeg et al., 2012). We therefore investigated whether there was variation in the response to our

pre-FMT bile acid combination across the five strains represented by our 10 isolates, and found that while all strains germinated in response to pre-FMT compared to post-FMT bile acids, this response did vary across strains, and in particular was more profound in NAP7 isolates (Fig. 4.10).

While little is known about the genetics and mechanisms of *C. difficile* germination, recent work has revealed that CspC, an analog of a serine protease involved in germination in *Clostridium perfringens*, may be the germinant receptor which senses bile acids in *C. difficile* (Francis et al., 2013). We therefore hypothesized that some of the variation in germination response to bile acids by our isolates might be due to variations in this receptor.

To test this hypothesis, we generated two sets of polymerase chain reaction (PCR) primers which targeted the published *cspC* gene sequence in *C. difficile* (Francis et al., 2013) and successfully sequenced the *cspC* gene from nine of our isolates (Table 4.4). Alignment of the protein sequences translated from the sequenced *cspC* genes revealed that while all sequences are highly similar, CspC sequences do tend to cluster by strain (Fig. 4.11A, Fig. 4.12). In particular, while sequences from the two successfully sequenced NAP7 isolates were identical, all other sequences were more similar to each other (99.1% or greater similarity), regardless of strain, than they were to NAP7 sequences (96.6 to 97.5% similarity) (Fig. 4.11B). We identified a total of 14 amino acid substitutions unique to the NAP7 isolates compared to all other isolates (Table 4.5), of which 7 were conservative, 3 were semi-conservative, and 4 were non-conservative. These findings suggest that there are identifiable mutations in the *C. difficile* germinant

receptor which may correspond to differences in germination in response to bile acids present in the feces of R-CDI patients.

DISCUSSION

Although fecal microbiota transplantation (FMT) is a highly effective treatment for R-CDI (Gough et al., 2011; Hamilton et al., 2012; van Nood et al., 2013; Youngster et al., 2014; Youngster et al., 2015), its acceptance into mainstream medicine requires further development and regulatory approval (Khoruts et al., 2014). Furthermore, some patients may not benefit from FMT, including those who require frequent courses of antibiotics for other infections. Therefore, there remains a need to develop non-antibiotic approaches to therapy for R-CDI. A mechanistic understanding of FMT has the potential to enable more rational development of such therapeutics.

Microbial metabolism of intracolonic bile acids provides a link between the transplantation of a healthy microbiome and durable recovery from CDI. The impact of bile acids on the *C. difficile* lifecycle has long been recognized. For example, taurocholate is routinely used in *C. difficile* culture media (Wilson, 1983; Arroyo et al., 2005), and both taurocholate and cholate are known to induce germination of the bacterium (Sorg and Sonenshein, 2008). In contrast, lithocholic acid, one of the dominant colonic secondary bile acids produced from primary bile acids via bacterial metabolism, is a known inhibitor of *C. difficile* germination (Sorg and Sonenshein, 2009; Sorg and Sonenshein, 2010).

The impact of bile acids on pathogenicity of *C. difficile* is further supported by animal models of the infection. The abundance of intracolonic secondary bile acids is markedly decreased in mice treated with antibiotics and susceptible to CDI and is significantly greater in mice resistant to CDI (Giel et al., 2010; Theriot et al., 2014;

Buffie et al., 2014; Koenigsknecht et al., 2014). These findings suggest that the types of bile acids present in the colon and the ability of *C. difficile* to respond to those bile acids might play an important role in this infection.

We previously showed that secondary bile acids are absent in the feces of patients with R-CDI syndrome, whereas primary bile acids are abundant (Weingarden et al., 2014). FMT promptly normalizes the fecal bile acid composition, decreasing primary bile acid concentrations and increasing secondary bile acids to levels found in healthy individuals. Therefore, we hypothesized that suppression of secondary bile acid metabolism in the colon by antibiotics creates an environment that is favorable for *C. difficile* germination and growth, and that restoration of normal secondary bile acid metabolism contributes to the clinical success of FMT in the treatment of R-CDI.

We tested these hypotheses here by measuring *C. difficile* spore germination and vegetative growth in the presence of mixtures of bile acids at concentrations found in pre-FMT and post-FMT feces of patients treated for R-CDI. Our results indicated that both germination and growth were inhibited in all tested, clinical isolates of *C. difficile* in the presence of post-FMT fecal bile acids (secondary bile acids) compared to pre-FMT bile acids (primary bile acids). However, recent work suggests that a model wherein primary bile acids promote *C. difficile* germination and secondary bile acids are inhibitory might be overly simplistic. One of the major primary bile acids in humans, chenodeoxycholic acid (CDCA), can prevent taurocholate-mediated spore germination (Sorg and Sonenshein, 2008; Sorg and Sonenshein, 2009). We confirmed these findings, although as previously reported we also found that high (non-physiological) concentrations of CDCA (2 mM) cause some strains of *C. difficile* to germinate (Heeg et al., 2012).

Importantly, however, CDCA is absorbed in the colon with far greater efficiency than cholate (Mekhjian et al., 1979), which diminishes its intraluminal impact relative to cholate derivatives. As we have shown, the resulting low CDCA concentration in pre-FMT feces cannot fully inhibit taurocholate- and cholate-induced *C. difficile* germination.

Furthermore, it is not clear that secondary bile acids cannot promote *C. difficile* germination. Deoxycholate, which is abundant in healthy donor and post-FMT feces, has been shown to be a germinant for *C. difficile* (Sorg and Sonenshein, 2008; Sorg and Sonenshein, 2009). Our results, however, suggest that many *C. difficile* strains do not germinate in response to physiologically relevant concentrations of deoxycholate (0.5 – 2 mM) as measured in post-FMT feces. Furthermore, compared to taurocholate, even greater concentrations of deoxycholate induce only modest germination (Sorg and Sonenshein, 2008; Theriot et al., 2014). Lithocholic acid, which is also elevated in post-FMT and donor feces, is not known to cause germination (Sorg and Sonenshein, 2010), in agreement with our present findings. Overall, our results suggest the combination of these two bile acids does not induce *C. difficile* germination, and emphasizes the importance of assessing the impact of combinations of bile acids which reflect the colonic environment in human patients, rather than individual bile acids.

One limitation of our study was the use of fecal measurements of bile acids, rather than intracolonic concentrations. The process of transforming primary to secondary bile acids by bacteria, 7α-dehydroxylation, is thought to be exclusively performed in the colon (Ridlon et al., 2006). It is likely that bile acid concentrations measured in the feces may underestimate the levels of primarily bile acids in the proximal colon. Furthermore, our results also demonstrated that post-FMT bile acids inhibit vegetative growth of *C.*

difficile, suggesting that even if some germination can occur in healthy or post-FMT individuals, proliferation of the bacteria would be limited.

Importantly, our findings were consistent across all 10 tested *C. difficile* isolates, including a representative of the NAP1 strain, which is considered hypervirulent and responsible for several epidemic outbreaks of CDI (McDonald et al., 2005). Since the germination response to CDCA varies among *C. difficile* strains (Heeg et al., 2012), it is necessary to investigate the response of a range of isolates to these bile acids. All of our tested *C. difficile* came from households of patients suffering from R-CDI who were treated with FMT, likely representing the strains responsible for disease in these patients. Therefore, our findings are likely to be directly applicable to future R-CDI patients.

Understanding the impact of intracolonic bile acids on the lifecycle of *C. difficile* allows investigators to rationally design novel and effective therapeutics. Recently, Buffie and colleagues demonstrated that inoculation with commensal bacteria that transform primary to secondary bile acids (7α-dehydroxylation) can prevent CDI in mice (Buffie et al., 2014). Additionally, a bile acid analog, a meta-benzene sulfonate derivative of cholate (CamSA), was found to prevent germination of *C. difficile* spores in a mouse colon assay and prevent symptoms in the infection model (Howerton et al., 2013a; Howerton et al., 2013b). Here, we explored the bile acid hypothesis based on data from a human system, including testing of a clinically-available secondary bile acid, UDCA. We demonstrated that UDCA prevents taurocholate-induced germination of spores from 10 clinical isolates of *C. difficile* across a range of concentrations, suggesting its potential as a therapeutic agent. Additionally, we demonstrated that UDCA can prevent vegetative

growth of *C. difficile*, suggesting that there is a second mechanism whereby this drug may help prevent recurrence of CDI.

Although these findings were overall consistent across our *C. difficile* isolates, we also found that the magnitude of response varied by PFGE type. In particular, isolates from the NAP7 PFGE type demonstrated a notably stronger response to the pre-FMT bile acid combination. This finding is not unexpected, given that other investigators have also demonstrated variation in *C. difficile* germination response to bile acids across a variety of ribotypes and isolates (Heeg et al., 2012). To explain the variation we found here, we sequenced the recently identified bile acid receptor, the serine protease CspC, from nine of our isolates (Francis et al., 2013). Our results indicated that although CspC sequence is highly similar across isolates, the CspC sequence of NAP7 isolates is somewhat more distinct, matching well with our germination results. Although the catalytic domain of *C. difficile* CspC is absent (Francis et al., 2013), limiting our ability to interpret the significance of mutations in NAP7 CspC compared to our other isolates, the presence of several non-conservative mutations suggests potential sites of interest which could be investigated further. Notably, NAP7 (ribotype 078), like NAP1, is known to be a hypervirulent strain of *C. difficile* and is associated with an increased mortality rate (Walker et al., 2013). It is therefore possible that this increased sensitivity to bile acid germinants, related to mutations in the germinant receptor, is a mechanism of increased virulence in this strain. In addition to fully exploring these mutations, sequencing of the CspC protein from additional isolates, both NAP7 and other PFGE types, will be necessary to fully understand the significance of these results.

Our understanding of the mechanisms behind FMT has been limited, which has reduced our ability to design novel therapeutics for individuals with R-CDI who do not meet inclusion criteria for or have failed FMT. Our results provide strong evidence that the observed shift in bile acid composition following FMT is an important mechanism underlying the therapeutic efficacy of the procedure. Furthermore, we have shown that UDCA inhibits *C. difficile* germination and growth and has potential as a novel therapeutic for R-CDI.

Table 4.1 - Average concentration of bile acids measured in patient feces before and after FMT and concentration used in bile acid combinations.

Bile acid	Concentration (mM)[a]	
	Pre-FMT	**Post-FMT**
Taurocholate (1^0)[b]	0.55	-[c]
Cholate (1^0)	1.45	-
Chenodeoxycholic acid (1^0)	0.37	-
Deoxycholate (2^0)[b]	-	1.24
Lithocholic acid (2^0)	-	0.95

[a]Mean fecal concentration, n=12 patients (Weingarden et al., 2014) and standard concentration used in this work.
[b]1^0 = primary bile acid, 2^0 = secondary bile acid.
[c]Bile acid undetected or below 1% of total bile acids; not used in experimental combination.

Table 4.2 - Characteristics of *Clostridium difficile* isolates used in this study.

PFGE type (isolate no.)[a]	*tcdC* deletion[b] (bp)	Binary toxin	Toxinotype (48)
NAP1	18	+	III
NAP2 (i)	0	-	0
NAP2 (ii)	0	-	0
NAP6 (i)	0	-	0
NAP6 (ii)	0	-	0
NAP6 (iii)	0	-	0
NAP7 (i)	39	+	V
NAP7 (ii)	39	-	V
NAP7 (iii)	39	-	V
NAP10	0	-	0

[a]PFGE = Pulsed field gel electrophoresis.
[b]*tcdC* deletion = length of deletion in *tcdC* gene.

Table 4.3 - Mean relative OD_{600} of spores from 10 isolates after 20 min exposure to bile acids.

	Bile Acid (mM)	PFGE type (isolate no.)[a]									
Bile acid		**NAP1**	**NAP2 (i)**	**NAP2 (ii)**	**NAP6 (i)**	**NAP6 (ii)**	**NAP6 (iii)**	**NAP7 (i)**	**NAP7 (ii)**	**NAP7 (iii)**	**NAP10**
Pre-FMT Bile Acids		0.90	0.93	0.88	0.90	0.88	0.86	0.60	0.65	0.62	0.88
SEM [i]		*0.02*	*0.08*	*0.02*	*0.03*	*0.01*	*0.01*	*0.01*	*0.01*	*0.02*	*0.01*
Post-FMT Bile Acids		1.01	1.00	1.02	1.09	1.07	1.02	0.96	1.04	1.05	1.11
SEM		*0.02*	*0.06*	*0.03*	*0.03*	*0.02*	*0.01*	*0.02*	*0.03*	*0.01*	*0.03*
BHIS[b]		0.97	1.03	1.10	1.01	1.10	1.07	1.10	0.97	1.08	0.94
SEM		*0.03*	*0.03*	*0.05*	*0.06*	*0.07*	*0.04*	*0.07*	*0.02*	*0.05*	*0.06*
TA[c]	0.5	0.66	0.73	0.76	0.90	0.84	0.89	0.70	0.69	0.62	0.67
SEM		*0.02*	*0.02*	*0.03*	*0.02*	*0.03*	*0.05*	*0.01*	*0.02*	*0.00*	*0.03*
	2	0.52	0.47	0.48	0.62	0.69	0.60	0.56	0.43	0.43	0.55
SEM		*0.01*	*0.03*	*0.01*	*0.02*	*0.01*	*0.01*	*0.02*	*0.03*	*0.01*	*0.01*
CA[d]	0.5	0.98	1.05	1.02	0.94	0.94	1.03	0.73	0.83	0.90	1.00
SEM		*0.01*	*0.08*	*0.04*	*0.01*	*0.03*	*0.03*	*0.10*	*0.04*	*0.06*	*0.04*
	1	0.89	1.01	0.97	1.03	0.94	0.93	0.7	0.66	0.79	0.90
SEM		*0.03*	*0.05*	*0.03*	*0.03*	*0.03*	*0.04*	*0.00*	*0.05*	*0.02*	*0.04*
	2	0.94	0.96	0.93	0.93	0.91	0.92	0.60	0.71	0.71	0.95
SEM		*0.02*	*0.04*	*0.01*	*0.04*	*0.04*	*0.03*	*0.01*	*0.04*	*0.02*	*0.04*
CDCA[e]	0.25	1.02	1.00	1.01	0.93	1.03	0.93	0.99	0.92	0.97	0.99
SEM		*0.06*	*0.06*	*0.06*	*0.09*	*0.04*	*0.02*	*0.03*	*0.02*	*0.03*	*0.03*
	0.5	1.00	1.08	1.00	0.95	0.94	0.97	1.09	0.98	0.91	1.05
SEM		*0.01*	*0.07*	*0.02*	*0.03*	*0.02*	*0.03*	*0.01*	*0.02*	*0.05*	*0.07*
	1	1.01	1.04	1.09	1.06	0.96	1.03	1.11	1.13	1.00	1.06
SEM		*0.02*	*0.03*	*0.01*	*0.04*	*0.03*	*0.01*	*0.04*	*0.04*	*0.07*	*0.07*

	2	0.91	0.90	1.07	0.98	0.96	0.98	0.83	0.97	0.94	0.77
SEM		*0.03*	*0.02*	*0.05*	*0.01*	*0.01*	*0.05*	*0.04*	*0.04*	*0.01*	*0.01*
CDCA+2 mM TA	0.25	0.57	0.61	0.56	0.64	0.64	0.70	0.57	0.59	0.62	0.57
SEM		*0.01*	*0.04*	*0.02*	*0.01*	*0.01*	*0.02*	*0.01*	*0.01*	*0.01*	*0.01*
	0.5	0.84	0.71	0.86	0.79	0.74	0.73	0.76	0.67	0.72	1.11
SEM		*0.07*	*0.05*	*0.03*	*0.07*	*0.01*	*0.01*	*0.01*	*0.03*	*0.02*	*0.11*
	1	1.05	0.86	1.02	1.01	0.85	0.91	0.87	0.87	0.83	1.10
SEM		*0.02*	*0.10*	*0.12*	*0.01*	*0.01*	*0.03*	*0.00*	*0.02*	*0.04*	*0.04*
	2	1.08	0.87	1.03	1.10	0.96	1.04	0.85	0.95	0.87	0.96
SEM		*0.04*	*0.06*	*0.04*	*0.01*	*0.04*	*0.01*	*0.03*	*0.03*	*0.01*	*0.05*
DCA[f]	0.5	1.04	1.06	1.02	0.93	0.89	0.93	0.72	0.77	0.92	1.03
SEM		*0.01*	*0.02*	*0.02*	*0.01*	*0.01*	*0.04*	*0.01*	*0.01*	*0.01*	*0.05*
	1	1.00	1.05	0.99	1.03	0.90	0.95	0.78	0.76	0.89	1.11
SEM		*0.01*	*0.04*	*0.05*	*0.02*	*0.05*	*0.04*	*0.08*	*0.07*	*0.03*	*0.06*
	2	1.12	1.00	0.83	0.97	0.86	0.80	0.98	0.71	0.85	1.05
SEM		*0.07*	*0.01*	*0.05*	*0.01*	*0.02*	*0.04*	*0.02*	*0.03*	*0.05*	*0.03*
LCA[g]	0.5	1.08	1.01	1.05	1.04	0.99	1.05	0.97	1.00	1.00	0.98
SEM		*0.02*	*0.01*	*0.02*	*0.03*	*0.02*	*0.04*	*0.03*	*0.02*	*0.02*	*0.04*
	1	1.05	1.06	1.03	1.02	1.00	1.00	1.04	1.03	0.94	0.96
SEM		*0.04*	*0.07*	*0.02*	*0.02*	*0.03*	*0.02*	*0.03*	*0.00*	*0.04*	*0.01*
	2	1.05	1.03	0.88	1.00	1.03	1.10	0.96	1.01	0.94	1.02
SEM		*0.06*	*0.11*	*0.11*	*0.04*	*0.03*	*0.05*	*0.04*	*0.02*	*0.03*	*0.03*
UDCA[h]+2 mM TA	0.5	1.05	1.02	0.98	0.84	0.76	0.78	0.63	0.55	0.60	0.96
SEM		*0.02*	*0.04*	*0.05*	*0.02*	*0.01*	*0.04*	*0.02*	*0.06*	*0.01*	*0.04*
	1	0.97	1.02	1.02	0.98	0.93	0.96	0.74	0.77	0.74	0.97
SEM		*0.05*	*0.04*	*0.02*	*0.01*	*0.03*	*0.05*	*0.03*	*0.04*	*0.02*	*0.06*
	2	1.04	1.16	1.05	1.05	1.06	0.96	1.00	0.91	0.99	1.03

SEM		*0.05*	*0.05*	*0.04*	*0.04*	*0.03*	*0.04*	*0.01*	*0.04*	*0.03*	*0.04*

[a]PFGE = Pulsed-field gel electrophoresis, [b]BHIS = BHI broth with yeast extract and 0.1% L-cysteine, [c]TA = taurocholate, [d]CA = cholate, [e]CDCA = chenodeoxycholic acid, [f]DCA = deoxycholate, [g]LCA = lithocholic acid, [h]UDCA = ursodeoxycholic acid.
[i]Bottom row of each group displays the standard error of the mean (SEM) for the mean listed in the cell above.

Table 4.4 – *C. difficile cspC* PCR primers used in this study.

Primer	Position on *cspC* gene (5' end)	Sequence (5' to 3')
Forward primer 1A	4	AACACGTATGCAACTATAACTG
Forward primer 1B	650	CAAATCCACCTCCAGAAGGT
Forward primer 1C	1300	GAAAATACTACTTATGCAATGAG
Reverse primer 1	2146	AAAATTCATAAGTCTAAATTATAATG
Forward primer 2A	22	ACTGGTACAGCAGCAGCA
Forward primer 2B	628	TTATGGGACCAAGAAGCAA
Forward primer 2C	1250	ATATAAGGTATTCTCCAGAC
Reverse primer 2	2167	ACTTTTAGCAATTTTACAAATAAA

Table 4.5 – Amino acid substitutions in CspC protein sequences of NAP7 isolates.

Position	Amino acid in most isolates	Amino acid in NAP7 isolates	Type of substitution
47	Leu	Ile	Conservative
59	Glu	Ala	Non-conservative
73	Asn	Asp	Conservative
83	Glu	Gly	Non-conservative
85	Asp	Glu	Conservative
179	Val	Ala	Semi-conservative
184	Ile	Val	Conservative
187	Gln	Lys	Conservative
206	Thr	Ile	Non-conservative
207	Asp	Gly	Semi-conservative
237	Lys	Arg	Conservative
250	Ser	Gly	Semi-conservative
338	Ser	Ala	Conservative
384	Ile	Thr	Non-conservative

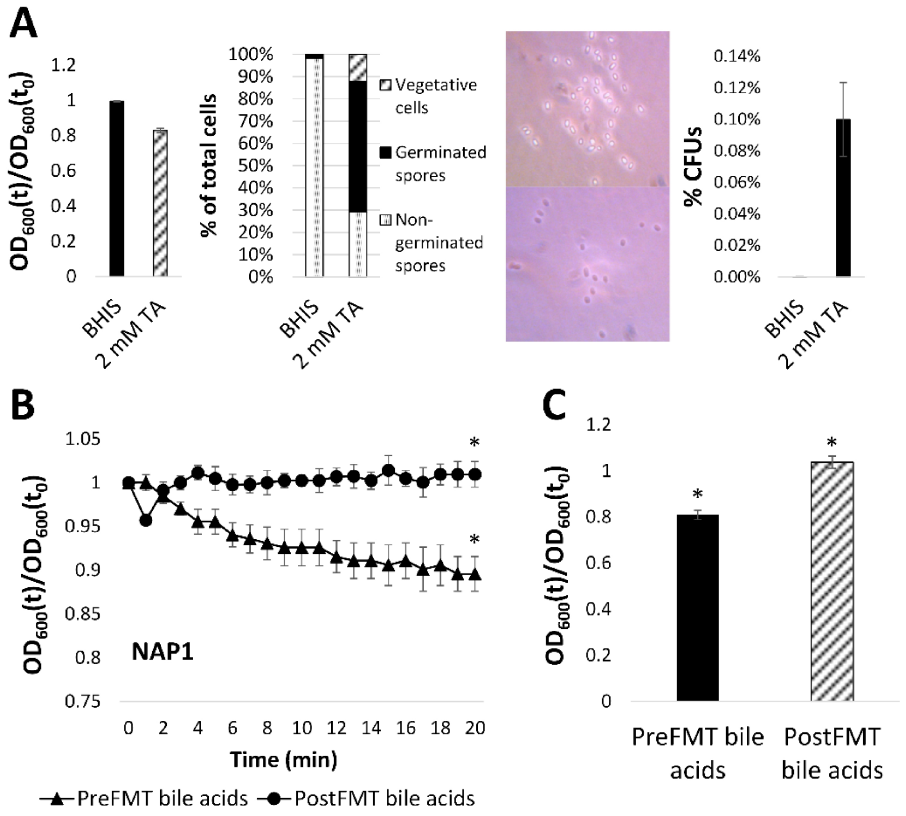


Figure 4.1 - *C. difficile* spores germinate in response to pre-FMT fecal bile acids. A) Decrease in relative OD_{600} after 20 min exposure to germinant (TA) corresponds to decrease in non-germinated spores (top) and appearance of germinated spores (bottom) and vegetative cells under phase-contrast microscopy, which in turn corresponds to outgrowth of spores and colony formation. BHIS: BHI with yeast extract and L-cysteine; 2 mM TA: BHIS with 2 mM taurocholate; CFUs: colony-forming units. $OD_{600}(t)/OD_{600}(t_0)$ = OD_{600} normalized to initial OD_{600} (relative OD_{600}); % CFUs = % of colonies formed from total number of cells. B) Relative OD_{600} of NAP1 spores following exposure to bile acids present before (▲) or after (●) FMT. C) Mean relative OD_{600} of spores from 10 isolates after 20 min of exposure to bile acids present before or after FMT. * = $p < 0.05$. Data represent mean ± SEM.

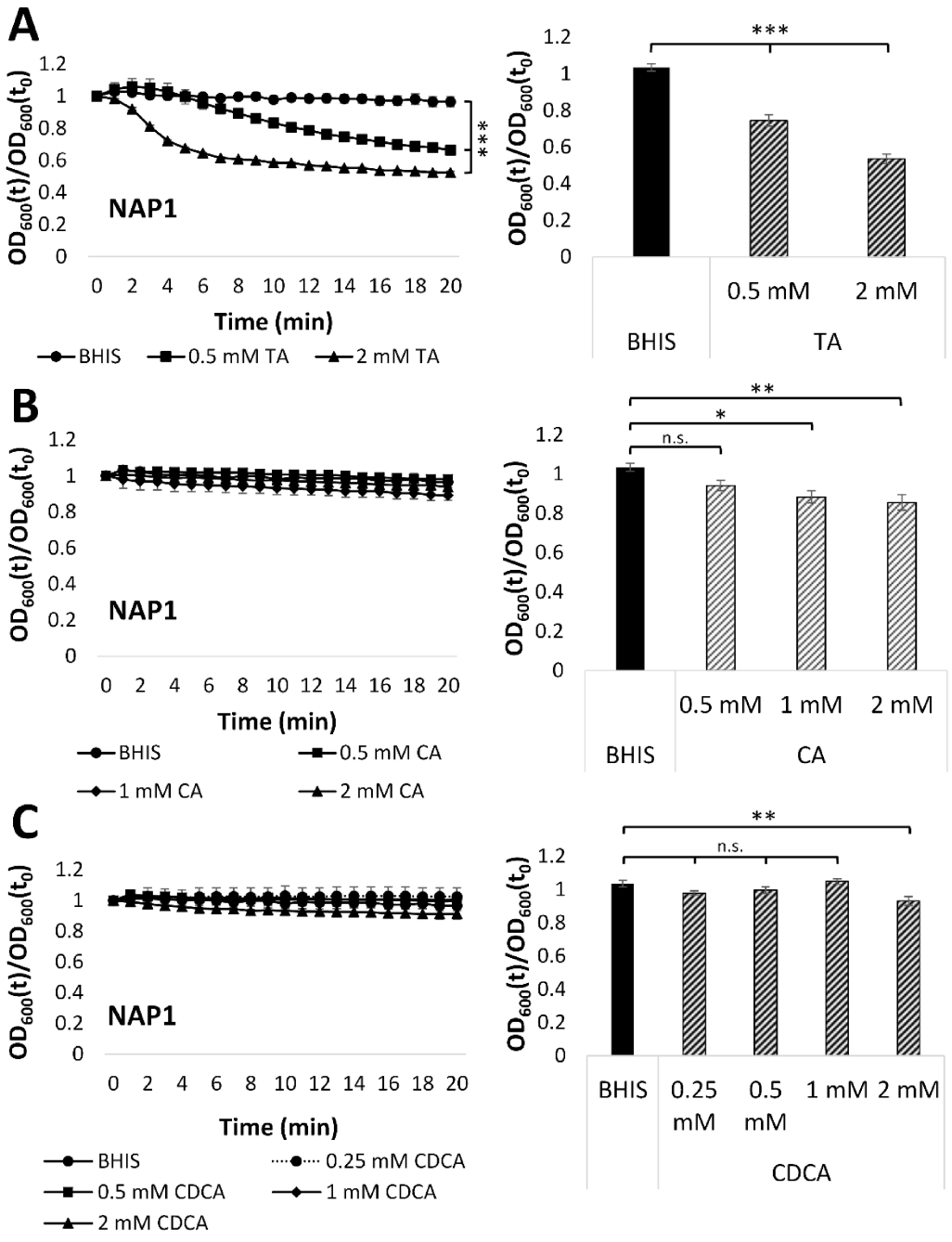


Figure 4.2 - *C. difficile* spores germinate in response to primary bile acids. A) (Left) Relative OD_{600} of NAP1 spores exposed to 0.5 mM (■) or 2 mM (▲) TA versus BHIS alone (●). (Right) Mean relative OD_{600} of spores from 10 isolates after 20 min exposure to 0.5 or 2 mM TA vs. BHIS alone. B) (Left) Relative OD_{600} of NAP1 spores exposed to

0.5 mM (■), 1 mM (♦), or 2 mM (▲) CA versus BHIS alone (●). (Right) Mean relative OD_{600} of spores from 10 isolates after 20 min exposure to 0.5, 1, or 2 mM CA vs. BHIS alone. C) (Left) Relative OD_{600} of NAP1 spores exposed to 0.25 mM (dashed line), 0.5 mM (■), 1 mM (♦), or 2 mM (▲) CDCA versus BHIS alone (●). (Right) Mean relative OD_{600} of spores from 10 isolates after 20 min exposure to 0.25, 0.5, 1, or 2 mM CDCA vs. BHIS alone. $OD_{600}(t)/OD_{600}(t_0)$ = OD_{600} normalized to initial OD_{600} (relative OD_{600}); *** = $p < 0.001$, ** = $p < 0.01$; * = $p < 0.05$, n.s. = non-significant. BHIS: BHI with yeast extract and L-cysteine; TA: taurocholate; CA: cholate; CDCA: chenodeoxycholic acid. Data represent mean ± SEM.

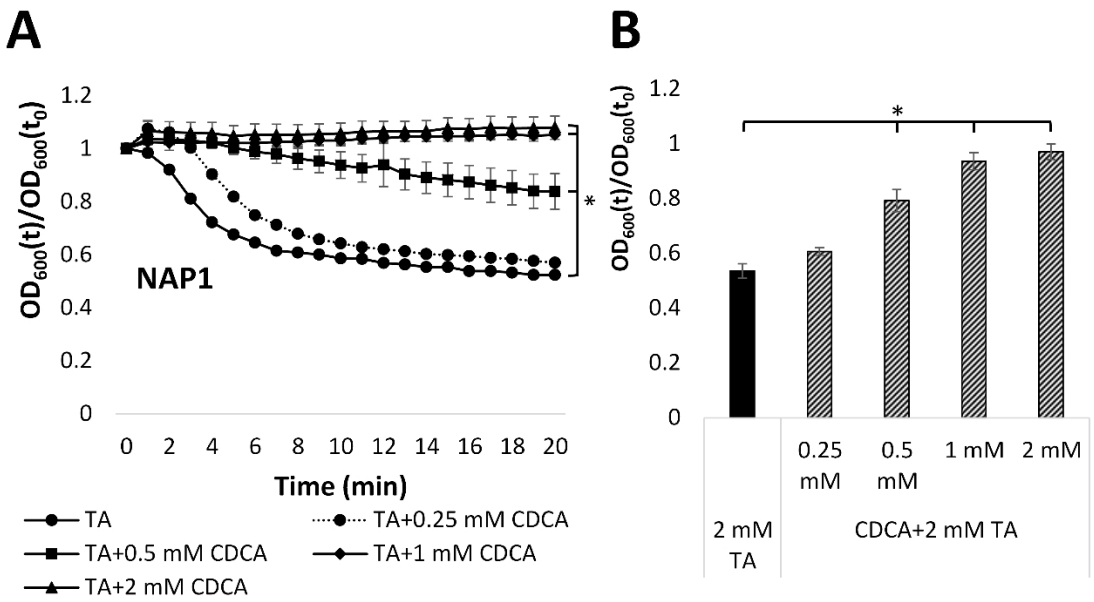


Figure 4.3 - CDCA inhibits taurocholate-mediated germination of *C. difficile.* A) Relative OD_{600} of NAP1 spores exposed to 0.25 mM (dashed line; ●), 0.5 mM (■), 1 mM (♦), or 2 mM (▲) CDCA and 2 mM TA versus TA alone (●). B) Mean relative OD_{600} of spores from 10 isolates after 20 min exposure to 0.25, 0.5, 1, or 2 mM CDCA and 2 mM TA vs. TA alone. $OD_{600}(t)/OD_{600}(t_0)$ = OD_{600} normalized to initial OD_{600} (relative OD_{600}); * = $p < 0.01$. BHIS: BHI with yeast extract and L-cysteine; TA: taurocholate; CDCA: chenodeoxycholic acid. Experiments performed in triplicate. Data represent mean ± SEM.

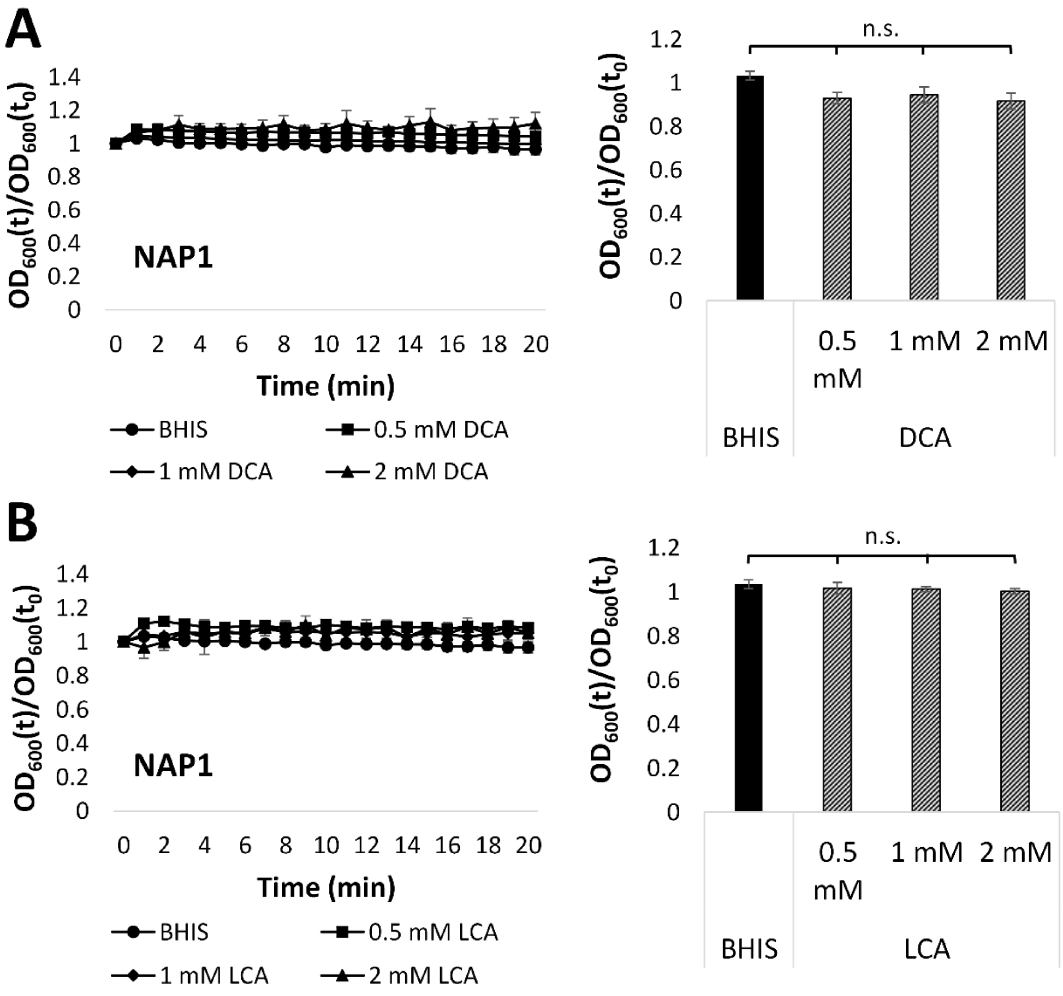


Figure 4.4 - *C. difficile* spores do not germinate in response to secondary bile acids. A) (Left) Relative OD_{600} of NAP1 spores exposed to 0.5 mM (■), 1 mM (♦), or 2 mM (▲) DCA versus BHIS alone (●). (Right) Mean relative OD_{600} of spores from 10 isolates after 20 min exposure to 0.5, 1, or 2 mM DCA vs. BHIS alone. B) (Left) Relative OD_{600} of NAP1 spores exposed to 0.5 mM (■), 1 mM (♦), or 2 mM (▲) LCA versus BHIS alone (●). (Right) Mean relative OD_{600} of spores from 10 isolates after 20 min exposure to 0.5, 1, or 2 mM LCA vs. BHIS alone. $OD_{600}(t)/OD_{600}(t_0)$ = OD_{600} normalized to initial OD_{600} (relative OD_{600}); n.s. = non-significant. BHIS: BHI with yeast extract and L-cysteine; DCA: deoxycholate; LCA: lithocholic acid. Data represent mean ± SEM.

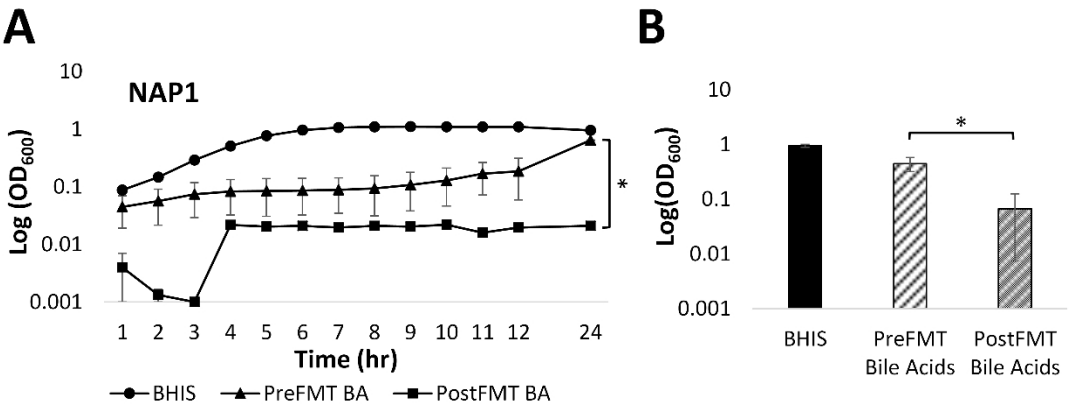


Figure 4.5 - Growth of vegetative *C. difficile* is inhibited by post-FMT bile acids. A) Hourly OD_{600} measurements for NAP1 cells in BHIS alone (●), BHIS with pre-FMT bile acids (▲), and BHIS with post-FMT bile acids (■). B) Mean OD_{600} at 24 hr across 10 isolates. BA: bile acids. * = $p<0.01$. Data represent mean ± SEM.

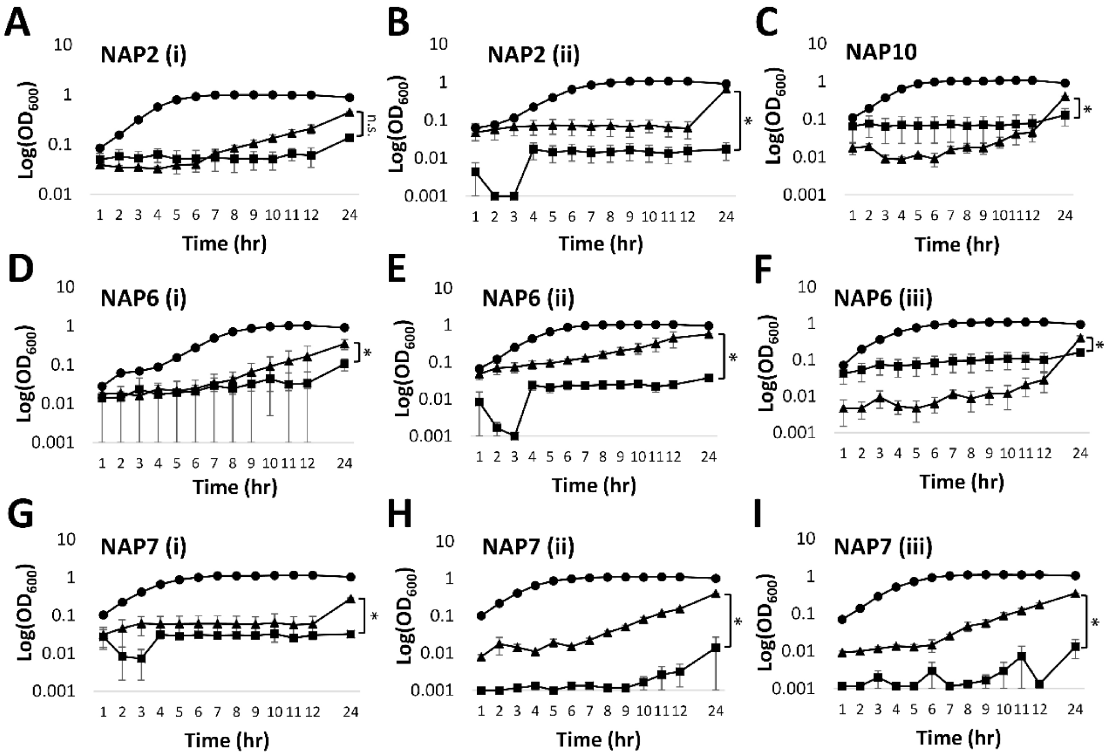


Figure 4.6 - Growth of vegetative *C. difficile* from all 10 isolates is inhibited by post-FMT bile acids. Hourly OD_{600} measurements for cells in BHIS alone (●), BHIS with PreFMT bile acids (▲), and BHIS with PostFMT bile acids (■). A-B) NAP2 isolates. C) NAP10 isolate. D-F) NAP6 isolates. G-I) NAP7 isolates. * = $p<0.01$; n.s. = non-significant. Experiments performed in triplicate. Data represent mean ± SEM.

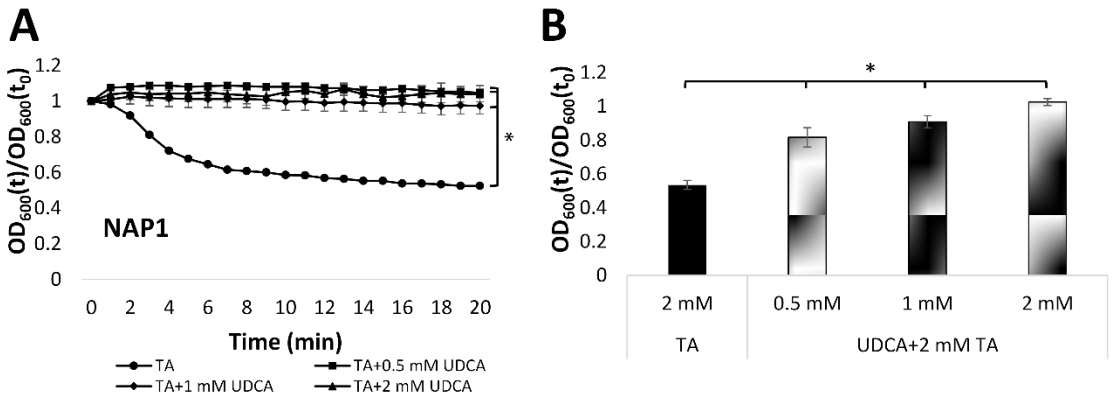


Figure 4.7 – UDCA inhibits *C. difficile* spore germination. A) Relative OD_{600} of NAP1 spores exposed to 0.5 mM (■), 1 mM (♦), or 2 mM (▲) UDCA in the presence of 2 mM TA vs. 2 mM TA alone (●). B) Mean relative OD_{600} of spores from 10 isolates after 20 min exposure to 0.5, 1, or 2 mM UDCA in the presence of 2 mM TA vs. 2 mM TA alone. $OD_{600}(t)/OD_{600}(t_0)$ = OD_{600} normalized to initial OD_{600} (relative OD_{600}); * = $p<0.01$. TA: taurocholate; UDCA: ursodeoxycholic acid. Data represent mean ± SEM.

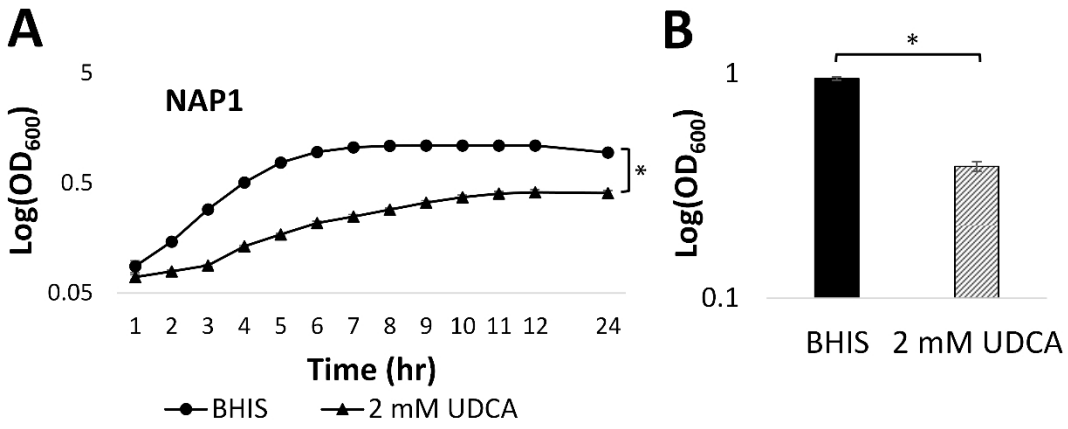


Figure 4.8 – UDCA inhibits vegetative growth of *C. difficile*. A) Hourly OD_{600} measurements for NAP1 cells in BHIS alone (●) or BHIS with 2 mM UDCA (▲). B) Mean OD_{600} at 24 hr across 10 isolates. UDCA: ursodeoxycholic acid. * = $p<0.0001$. Data represent mean ± SEM.

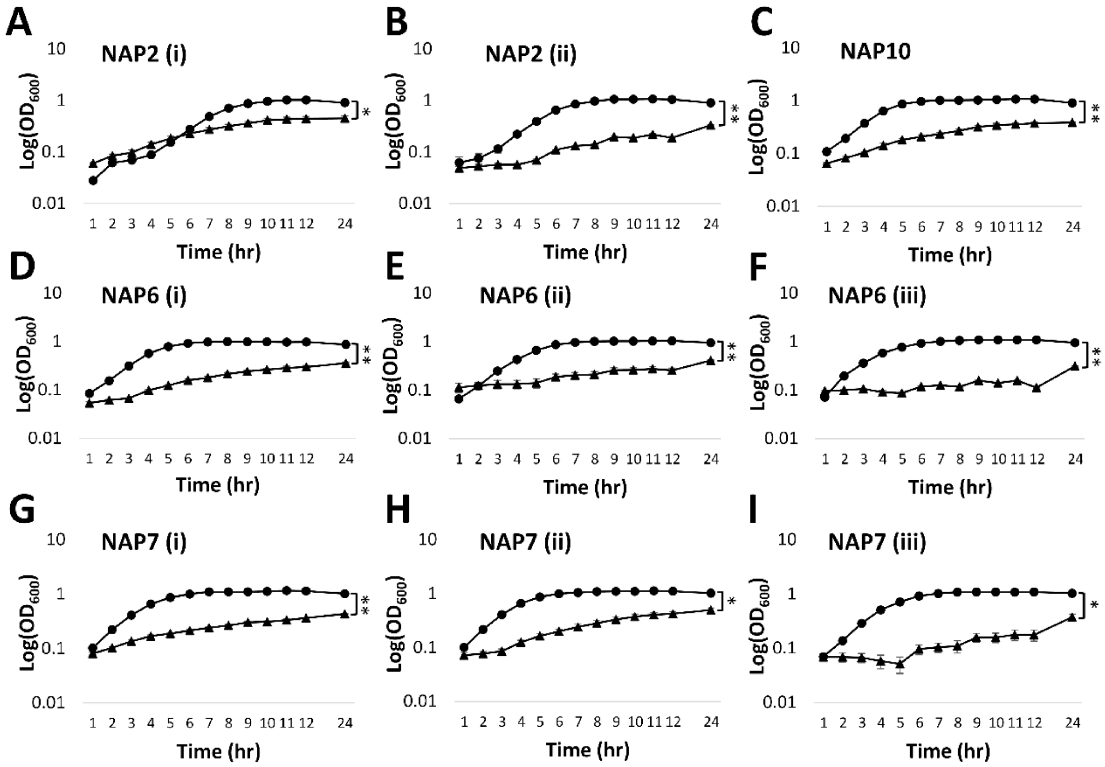


Figure 4.9 – Growth of all *C. difficile* isolates is inhibited by UDCA. Hourly OD_{600} measurements for cells in BHIS alone (●) or BHIS with 2 mM UDCA (▲) A-B) NAP2 isolates. C) NAP10 isolate. D-F) NAP6 isolates. G-I) NAP7 isolates. * = $p<0.001$; ** = $p<0.0001$ Experiments performed in triplicate. Data represent mean ± SEM.

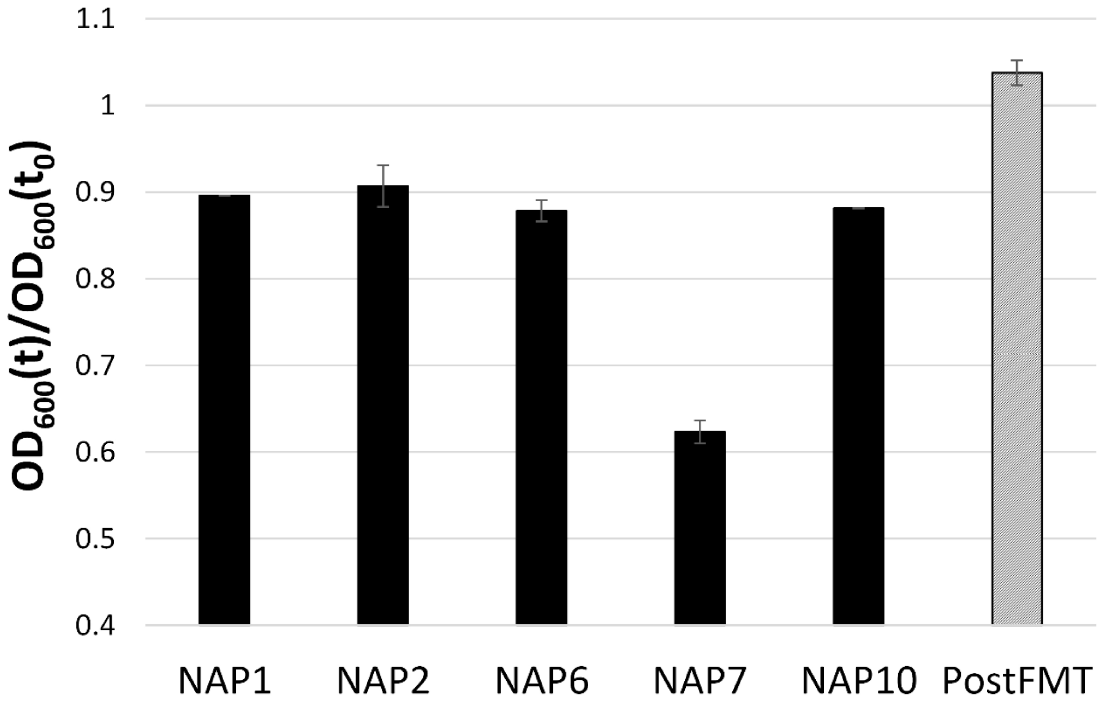
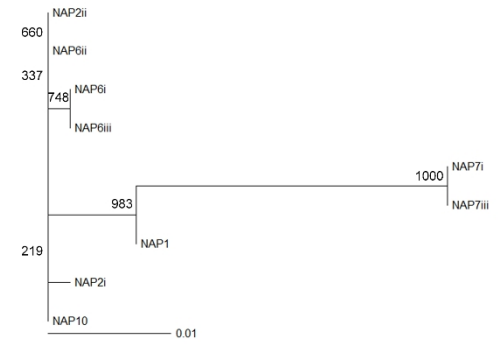


Figure 4.10 – Germination of *C. difficile* spores varies by strain. Mean relative OD_{600} of spores from the five PFGE types used in this study when exposed to pre-FMT bile acids (black bars) or of spores from all isolates when exposed to post-FMT bile acids (grey bar) for 20 min. $OD_{600}(t)/OD_{600}(t_0)$ = OD_{600} normalized to initial OD_{600} (relative OD_{600}). Data represent mean ± SEM across isolates within PFGE type.

A



B

	NAP7i	NAP7iii	NAP1	NAP6i	NAP6iii	NAP2i	NAP2ii	NAP6ii	NAP10
NAP7i	100	100	97.49	96.59	96.59	96.59	96.77	96.77	96.77
NAP7iii	100	100	97.49	96.59	96.59	96.59	96.77	96.77	96.77
NAP1	97.49	97.49	100	99.10	99.10	99.10	99.28	99.28	99.28
NAP6i	96.59	96.59	99.10	100	100	99.64	99.82	99.82	99.82
NAP6iii	96.59	96.59	99.10	100	100	99.64	99.82	99.82	99.82
NAP2i	96.59	96.59	99.10	99.64	99.64	100	99.82	99.82	99.82
NAP2ii	96.77	96.77	99.28	99.82	99.82	99.82	100	100	100
NAP6ii	96.77	96.77	99.28	99.82	99.82	99.82	100	100	100
NAP10	96.77	96.77	99.28	99.82	99.82	99.82	100	100	100

Figure 4.11 – CspC sequences primarily cluster by PFGE type. A) Boot-strapped phylogenetic tree of CspC sequences generated by UPGMA method. Boot-strap values are indicated at each node. B) Percent identity matrix of CspC sequences from different isolates. Roman numerals indicate isotype number within each PFGE type. NAP: North American pulsed-field gel electrophoresis (PFGE) type.

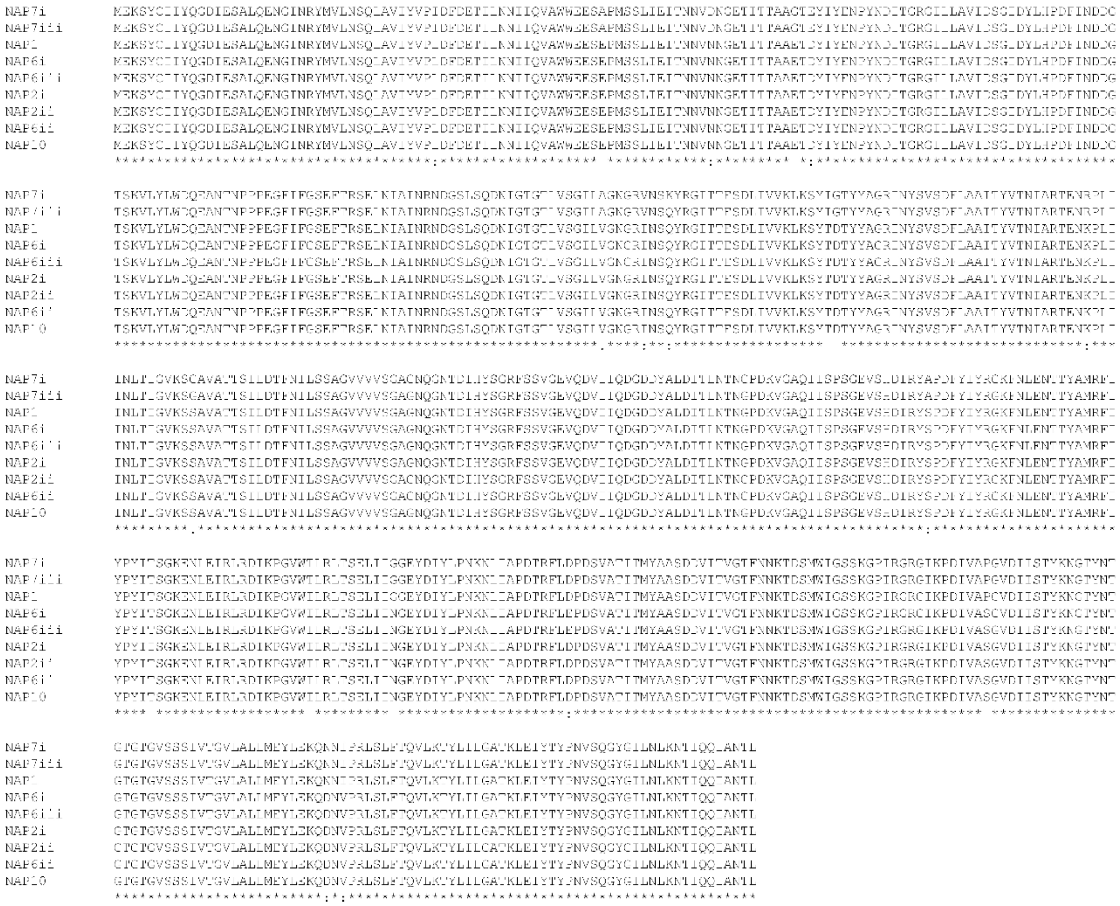


Figure 4.12 – Alignment of *C. difficile* CspC protein sequences. Protein sequences from 9 isolates. Roman numerals represent isolate number within PFGE type. * = conserved residue; : = conservative mutation; . = semi-conservative mutation; blank = non-conservative mutation. NAP: North American pulsed-field gel electrophoresis (PFGE) type.

Chapter 5

C7-Sulfated Ursodeoxycholic Acid as a Novel Therapy to Prevent *Clostridium difficile* Infection

SUMMARY

Clostridium difficile infection (CDI) is the most common nosocomial infection in the US, but unfortunately standard antibiotic therapy is not effective in a number of patients, creating a need for novel, non-antibiotic treatments. We recently demonstrated that a potential mechanism behind a powerful emerging treatment for CDI, fecal microbiota transplantation (FMT), is a shift in fecal bile acid composition. Specifically, restoration of bacterial secondary bile acid metabolism in the feces following FMT prevents germination and growth of *C. difficile*. Furthermore, we demonstrated that a clinically available secondary bile acid, ursodeoxycholic acid (UDCA), can inhibit *C. difficile* germination and growth. Because UDCA may be of limited therapeutic utility due to its absorption into enterohepatic circulation, here we examined whether synthetic C7-sulfated UDCA (7-SUDCA), which is not significantly absorbed and achieves a high concentration in the colon, could inhibit *C. difficile* germination. Using an *in vitro* assay for germination and a mouse model of CDI, we determined that 7-SUDCA can inhibit *C. difficile* germination *in vitro* and both reduce pathology and delay the appearance of *C. difficile* in the feces *in vivo.* These findings suggest that 7-SUDCA is a promising non-antibiotic therapy for the prevention of CDI.

INTRODUCTION

Clostridium difficile infection (CDI) is an extremely common nosocomial infection, responsible for nearly half a million cases of diarrhea, and nearly 30,000 deaths, in the United States each year (Lessa et al., 2015). Unfortunately, standard antibiotic therapy often fails to cure CDI, as symptoms return in 20-30% of patients following the cessation of antibiotics (Kelly and Lamont, 2008; Vardakas et al., 2012; Lessa et al., 2015). Once an individual has experienced a single such recurrence of the infection, the chances for further recurrence can be as high as 65% or greater (Khoruts and Sadowsky, 2011). Often, these patients eventually fall into vicious cycles characterized by repeated antibiotic treatment and recurrence of disease, a state known as recurrent CDI syndrome (R-CDI) (Borody and Khoruts, 2011).

For patients with R-CDI, therapy options are limited. Although these patients are typically refractory to further antibiotic treatment, fecal microbiota transplantation (FMT) is emerging as a highly effective therapy for R-CDI (Yoon and Brandt, 2010; Hamilton et al., 2012; van Nood et al., 2013). However, FMT may not be the best treatment option for all patients with R-CDI; for example, patients who require frequent antibiotic use for other conditions often do not meet the criteria for FMT. We have recently demonstrated that a shift in fecal bile acid composition as a result of the procedure may be a mechanism behind FMT (Weingarden et al., 2014; Chapter 4). Specifically, the bile acids present in the feces before FMT, typically primary bile acids produced by the liver, induce *C. difficile* germination and allow growth of vegetative cells, while the bile acids present after FMT, typically secondary bile acids which are transformed from primary

bile acids by native colonic bacteria (Ridlon et al., 2006), do not induce germination and inhibit growth. Furthermore, recent work has indicated that treatment with bile acid-transforming bacteria can limit *C. difficile* infection in mice (Buffie et al., 2014). These findings suggest that manipulation of fecal bile acid composition is a viable strategy for uncovering novel treatments for R-CDI.

In particular, recent work suggests that pharmacological manipulation of fecal bile acids is a promising avenue of CDI treatment. A bile acid analog, a meta-benzene sulfonate derivative of cholate (CamSA), was found to prevent germination of *C. difficile* spores in a mouse colon assay and prevent symptoms in a model of infection (Howerton et al., 2013a; Howerton et al., 2013b). Furthermore, our work and the work of others has indicated that ursodeoxycholic acid (UDCA), a minor human secondary bile acid clinically available as a therapeutic, can also inhibit *C. difficile* germination and growth (Chapter 4, Sorg and Sonenshein, 2010). However, despite the promise of UDCA, it is well-absorbed in the small intestine, limiting its intracolonic concentration (Rodrigues et al., 1995; Hofmann and Hagey, 2008), and therefore it may have limited *in vivo* potential. In contrast, a derivative of UDCA, C7-sulfated UDCA (7-SUDCA), is poorly absorbed in the small intestine and may have potential as a new treatment for CDI (Rodrigues et al., 1995).

Here we demonstrate that 7-SUDCA may be a novel therapeutic for CDI. We show that 7-SUDCA inhibits *C. difficile* germination *in vitro*, and developed a mouse model of CDI to investigate whether 7-SUDCA can prevent *C. difficile* infection *in vivo*. Our results indicate that in this model, 7-SUDCA can prevent weight loss associated with

CDI and delays the appearance of *C. difficile* colony-forming units (CFUs) in the feces. These preliminary findings suggest that 7-SUDCA is a potential novel therapy for CDI.

MATERIALS AND METHODS

Synthesis of C7-sulfated UDCA

C7-sulfated UDCA (7-UDCA) was synthesized as described by Setchell, 1996. Imidazole (3.84 g, 56.4 mmol) and tert-butyldimethylsilyl chloride (1.86 g, 12.3 mmol) were added to a solution of UDCA (1.98 g, 5.04 mmol) in DMF (1.5 mL) and pyridine (0.75 mL) at 0 °C. The solution was stirred for 30 min at RT, diluted with ice water, and extracted with ethyl acetate. The organic layer was dried under Na_2SO_4 and concentrated under reduced pressure. The resulting residue was purified by silica gel column (25% EtOAc/hexanes) and recrystallized from ethanol to furnish a white solid (1.315 g). 627 mg of this material was dissolved in 9 mL pyridine and placed in an ice bath. Chlorosulfonic acid (0.6 mL) was added dropwise and then the mixture was heated to 50°C for 30 min. After cooling to RT, water (300 mL) was added and the resulting precipitate collected by filtration (597 mg). 60.5 mg of this solid was suspended in acetone (2 mL) and concentrated HCl added (150 μL). After 30 min., the mixture was concentrated under reduced pressure, run through a C_{18} cartridge (1 gram) washing with methanol, and concentrated under reduced pressure. The residue was dissolved in methanol (0.5 mL) and NaOMe (0.5 mL of 0.5 M solution in methanol) was added. Cold ether (10 mL) was added and the resulting fine precipitate collected by filtration. The solid was redissolved in methanol (1.0 mL) and reprecipitated with cold ether (10 mL) to furnish 14 mg of C7-sulfated UDCA as a disodium salt.

C. difficile spore isolation

C. difficile cells from frozen stocks were inoculated into CCFB medium and grown anaerobically at 37°C for 48 hr. Cultures were plated onto brain heart infusion with 5 g/L yeast extract and 0.1% L-cysteine (BHIS) and grown for 7 d at 37°C under anaerobic conditions. Following Sorg and Sonenshein, 2010, colonies from each plate were scraped into 1 mL of ice-cold water and incubated at 4°C overnight to release spores. A 3 mL suspension was loaded onto 10 mL of 50% (w/v) sucrose in a 15 mL conical tube and centrifuged in a swinging bucket rotor at 3200 x *g* for 20 min at 4°C. Sucrose and vegetative cells above the spore pellet were removed, and the pellet was washed 5 times in ice-cold water to remove remaining sucrose. Spores were examined under phase-contrast microscopy to determine purity; spore samples with >99% purity (<1% vegetative cells) were stored at 4°C.

C. difficile spore germination

Germination assays were done as previously described by Sorg and Sonenshein, 2010. Spores were heated to 65°C for 30 min and inoculated into BHIS, with or without bile acids, within an anaerobic bag flushed and filled with N_2 gas. OD_{600} was measured initially ($OD6_{00}(t_0)$) and every minute for 20 min ($OD_{600}(t)$) using an EL808 Microplate Reader (Biotek Instruments, Inc., Winooski, VT). Relative OD_{600} for each time point was calculated as $OD_{600}(t)/OD_{600}(t_0)$. Experiments were performed in triplicate.

Infection of mice with C. difficile

Following the protocol of Theriot et al., 2011, 8 week old female C57BL/6 mice were given 0.5 mg/mL cefoperazone in drinking water for 10 days, changing the water every

2-3 days to keep the antibiotic fresh. After 10 days, cefoperazone was removed, and mice were allowed to recover for 2 days prior to infection. Mice were infected with 2 x 10^5 spores of a NAP1 isolate (Chapter 4) via oral gavage, while controls were given PBS. Fecal pellets were collected prior to antibiotic treatment, at the conclusion of antibiotic treatment, and daily following infection for 3 or 5 days. After 3 or 5 days, mice were sacrificed and cecal contents were collected. Mice were weighed prior to infection and each day following infection until sacrifice.

Administration of 7-SUDCA

In the first experiment, 7-SUDCA (15 mg/mL) was originally administered via drinking water, for 2 days prior to infection. Additionally, 37.5 mg of 7-SUDCA in 100 µL sterile water was administered to mice the day prior to infection, 4 hours prior to infection, and on days 2-4 following infection, via oral gavage. In the second experiment, mice were given two gavages per day of 18.75 mg of 7-SUDCA per gavage one day prior to infection and the day of infection, then given one gavage per day on days 1-2 following infection.

Assessment of C. difficile growth from pellets

Fecal pellets from each mouse were divided into two portions, weighed, and suspended in 200 µL sterile water. One set of fecal pellets was heated at 65°C for 30 min to isolate spores. Suspended fecal material was diluted by 10^3, 10^4, and 10^5, and plated on taurocholate-cycloserine-cefoxitin-fructose agar (TCCFA) (Bliss et al., 1997). Plates

were incubated overnight at 37°C under anaerobic conditions before calculating the number of colony-forming units (CFUs). CFUs were normalized to g feces.

Approval for mouse studies

This study was approved by the University of Minnesota Institutional Animal Care and Use Committee (IACUC 1309-30905A) and the Institutional Biosafety Committee (IBC 1310-30990H).

RESULTS

7-SUDCA prevents C. difficile germination in vitro

Although we have shown that UDCA can inhibit *C. difficile* germination *in vitro* (Chapter 4), it is unlikely to be useful for R-CDI in the majority of patients due to poor colonic delivery (Rodrigues et al., 1995, Hofmann and Hagey, 2008). To overcome this limitation, we synthesized a C7-sulfated derivative of UDCA (7-SUDCA), which is poorly absorbed in the small intestine (Rodrigues et al., 1995), and tested whether it could also inhibit germination of *C. difficile*. To investigate the potential of 7-SUDCA as an inhibitor of *C. difficile* germination, we used an established spectrophotometric assay which relies on the refractive nature of bacterial spore coats (Sorg and Sonenshein, 2008; Sorg and Sonenshein, 2009; Sorg and Sonenshein, 2010; Heeg et al., 2012). A decrease in OD_{600} over time indicates loss of this spore coat and therefore initiation of germination (Moir and Smith, 1990). We have previously used and validated this assay to confirm that this decrease in OD_{600} corresponds both to spore coat loss and ability of the spores to subsequently form colonies (Chapter 4).

To investigate the effectiveness of 7-SUDCA, we tested its ability to inhibit germination of spores from a NAP1 hypervirulent strain of *C. difficile* which we had previously isolated and described (Chapter 4). These spores were exposed to a known germinant, taurocholate, with or without different concentrations of 7-SUDCA. When spores were exposed to ≥2 mM concentrations of 7-SUDCA in the presence of taurocholate, germination was significantly ($p<0.01$) inhibited compared to spores exposed to taurocholate alone (Fig. 5.1A, Table 5.1). Furthermore, when spores from a

set of 10 isolates including the NAP1 strain, all previously described (Chapter 4), were exposed to taurocholate with or without 7-SUDCA, 2 mM or greater concentrations of the drug significantly ($p<0.01$) inhibited germination (Fig. 5.1B, Table 5.1). These results indicate that 7-SUDCA can inhibit germination of a variety of *C. difficile* strains, and that it may have potential as a novel therapeutic to prevent recurrence of CDI.

Establishing a mouse model of CDI

With these promising *in vitro* results, we next moved towards testing 7-SUDCA in an *in vivo* model. We used a mouse model of *C. difficile* infection based on the model described by Theriot and colleagues (Theriot et al., 2011). In this model, mice are given the antibiotic cefoperazone (0.5 mg/mL) for 10 days, followed by two days recovery off the antibiotic before infection with 2×10^5 *C. difficile* spores via oral gavage. Theriot et al., 2011, have demonstrated that in this model mice maintain the bacterium in their stool and lose weight across 2-5 days post-infection with a variety of *C. difficile* strains, although the severity of disease varies across these strains. One such strain was a clinical isolate of the NAP1 pulsed-field gel electrophoresis/027 ribotype, similar to the NAP1 isolate used in our *in vitro* work above and elsewhere (Chapter 4). We therefore used our NAP1 isolate for infection when developing our model (Fig. 5.2).

Following infection with this NAP1 isolate, mice were weighed daily, and daily fecal pellets were collected and plated on selective taurine-cycloserine-cefoxitin-fructose agar (TCCFA) to enumerate *C. difficile* colony-forming units (CFUs) in the feces. At 2 and 3 days post-infection, mice infected with the NAP1 isolate weighed significantly less than control uninfected mice (Fig. 5.3). Furthermore, mice maintained between 10^6 and

10^8 *C. difficile* CFUs per gram feces up to 5 days following infection (Fig. 5.4). These findings suggest that this model is capable of replicating CDI in human patients and therefore represents a useful model for testing novel therapies for this disease.

7-SUDCA prevents weight loss associated with CDI and delays appearance of CFUs in stool

Using our established model of CDI, we sought to investigate whether 7-SUDCA could prevent infection. Initially, mice were given 7-SUDCA in drinking water (15 mg/mL), but the drug was found to be unpalatable. Instead, after 1 day of recovery following antibiotic treatment, mice were given 37.5 mg of 7-SUDCA via oral gavage. This gavage was repeated the day of infection and 2-4 days post-infection. Both weight and CFU counts were tracked daily in these mice following infection (Fig. 5.2).

Unlike control mice infected with *C. difficile*, mice given 7-SUDCA before and after infection weighed significantly ($p<0.05$) more at 2, 3, and 5 days post-infection (Fig. 5.3). Furthermore, on the first day following infection, there were substantially fewer colonies formed by *C. difficile* spores (Fig. 5.4A) and total cells (Fig. 5.4B) in mice treated with 7-SUDCA compared to controls; in fact, the number of CFUs from one mouse was at or below the limit of detection. However, perhaps because mice were not treated with drug on the first day following infection, the number of CFUs on subsequent days was comparable to the number of CFUs from control mice. Despite this, however, these are promising preliminary results which suggest that 7-SUDCA can both decrease severity of disease and delay the appearance of *C. difficile* in the feces following infection.

Improving delivery of 7-SUDCA increases stress due to gavage but decreases C. difficile appearance in stool

Although our results above indicated that 7-SUDCA could partially inhibit infection with *C. difficile* spores, we next investigated whether improving drug delivery could further ameliorate disease. Because the gavage to rectum transit time of mice is typically 6-7 hours (Padmanabhan et al., 2013), a single gavage per day may not insure that colonic levels of 7-SUDCA remain at a high enough concentration to prevent *C. difficile* germination. Therefore, we repeated the previous experiment, giving two gavages with half the amount of drug (18.75 mg per gavage, vs. 37.5 mg) per day (Fig. 5.5).

Unfortunately, we found here that the stress of additional gavage masked the effects of treatment on mouse weight. Mice given both the drug and *C. difficile* spores did not weigh significantly more than control infected mice up to 3 days post-infection (Fig. 5.6). However, mice given drug but not infected also did not weigh significantly more than control infected mice, suggesting that the beneficial effects on weight seen in the previous experiment were masked here by the stress of additional gavage.

Although the effects on weight were less clear, treatment with 7-SUDCA still delayed the appearance of *C. diffiicile* in the stool. Mice treated with 7-SUDCA continued to have fewer CFUs of *C. difficile* in feces on day 1 post-infection compared to untreated mice, particularly fewer total cells (vegetative cells and spores) (Fig. 5.7). Notably, of the four animals tested on 1 d.p.i., the numbers of *C. difficile* CFUs in feces (total cells and spores) were below the limit of detected in three animals. Once again,

these results suggest that 7-SUDCA can ameliorate the effects of *C. difficile* infection and may therefore be a novel therapy for R-CDI.

DISCUSSION

Clostridium difficile infection (CDI) is the most common nosocomial infection in the US (Zilberberg et al., 2008; Miller et al., 2011; Dubberke and Olsen, 2012; Magill et al., 2014), but unfortunately standard antibiotic therapy frequently fails to clear the infection, resulting in a recurrence of disease in 20-30% of patients (Kelly and Lamont, 2008; Borody and Khoruts, 2011; Louie et al., 2011; Surawicz and Alexander, 2011). Although fecal microbiota transplantation (FMT) is becoming widely accepted as a therapy for patients with recurrent CDI (R-CDI) (Yoon and Brandt, 2010; Hamilton et al., 2012; Kassam et al., 2013), there remains a need to develop non-antibiotic therapeutic options for patients who cannot have an FMT or who fail to recover following the procedure.

Recently we demonstrated that ursodeoxycholic acid (UDCA) is a potential novel therapy for CDI which inhibits both germination and vegetative growth of *C. difficile* (Chapter 4). Unfortunately, clinical efficacy of UDCA in R-CDI is hindered by its efficient absorption into the enterohepatic circulation, preventing delivery to the colon, the site of infection (Rodrigues et al., 1995; Hofmann and Hagey, 2008). Therefore, here we tested inhibition of *C. difficile* germination by a minimally absorbed UDCA derivative, C7-sulfated UDCA (7-SUDCA). Taurocholate-induced *C. difficile* spore germination was inhibited by 7-SUDCA at a range of concentrations below that measured in the colon in an animal model (Rodrigues et al., 1995). These results suggest that 7-SUDCA may be a potential non-antibiotic therapeutic for R-CDI.

To explore the potential of 7-SUDCA as a therapy for R-CDI, we developed a mouse model based on the model of Theriot et al., 2011. Our work with this model demonstrated that mice infected with *C. difficile* spores following 10 days of cefoperazone treatment resulted in significant weight loss relative to uninfected animals as well as growth of over 10^6 colony-forming units (CFUs) of *C. difficile* per g feces in these animals from the day after infection until sacrifice (5 d post-infection). This model, therefore, represents a model of productive infection useful for testing whether 7-SUDCA can prevent or limit *C. difficile* infection *in vivo*.

Treatment with 7-SUDCA using this model indicated that the drug has potential as a novel therapy for CDI. Specifically, 7-SUDCA prevented weight loss associated with infection and delayed the appearance of *C. difficile* in the stool. These findings indicate that unlike UDCA, 7-SUDCA may be useful for preventing recurrence of CDI in patients with normal gastrointestinal anatomy. Unfortunately, 7-SUDCA was found to be poorly palatable to mice, resulting in decreased delivery of drug (and the loss of two animals). Mice were not treated 1 day post-infection, possibly leading to increased appearance of *C. difficile* in the stool after this point. Additionally, once-daily delivery of 7-SUDCA may not be sufficient to provide continuous protection against *C. difficile*, as the gastrointestinal transit time of mice is only ~6 hours following gavage (Padmanabhan et al., 2013). However, multiple gavages per day to insure higher concentrations of drug in the colon may substantially increase stress and therefore mask beneficial effects on weight.

Additional work is therefore necessary to fully test whether 7-SUDCA is an effective therapy to prevent CDI. Further improvements to drug delivery will be

necessary to assess the effects of the drug on CDI without causing additional stress to the animals, which obscures the effects of the drug. Furthermore, additional analysis of this current experiment is in progress, including histopathology of animal colons following sacrifice, 16S rRNA gene analysis of the feces and cecal contents to track bacterial communities of this model, and bile acid analysis of feces and cecal contents to track other germinants and germination inhibitors as well as to ensure that 7-SUDCA is reaching the site of infection.

7-SUDCA is not the first bile acid analog tested as a treatment for CDI in a mouse model. Recent work by Howerton and colleagues (2013) has demonstrated that an analog of cholic acid, CamSA, is capable of decreasing symptoms of CDI and decreasing the number of vegetative cells in animal feces. This work tested efficacy when drug was given 24 hr prior to infection, 1 hr after infection, and 24 hr after infection. In contrast, we saw similar results when drug was delivered 24 and 4 hours prior to infection, suggesting that we may see even more promising results if 7-SUDCA is given immediately following infection and on the first day following infection. Importantly, CamSA is not water soluble and was given dissolved in dimethyl sulfoxide (DMSO), while 7-SUDCA is water soluble and was delivered to mice in water. The use of DMSO may limit the ability of CamSA to be translated effectively into human patients, while 7-SUDCA has no such limitation.

Although the results presented here are limited, they indicate that 7-SUDCA may be a novel therapy to prevent recurrence of CDI. *In vitro*, 7-SUDCA prevents germination of *C. difficile*, while *in vivo* 7-SUDCA abrogates weight loss of animals and delays the appearance of *C. difficile* in the stool. Overall, these are promising findings

that suggest with additional work 7-SUDCA may be a useful and translatable treatment for *Clostridium difficile* infection.

Table 5.1 - Mean relative OD_{600} of spores from 10 isolates after 20 min exposure to 7-SUDCA and 2 mM taurocholate.

7-SUDCA[a] concentration (mM)		PFGE type (isolate no.)[b]									
		NAP1	**NAP2 (i)**	**NAP2 (ii)**	**NAP6 (i)**	**NAP6 (ii)**	**NAP6 (iii)**	**NAP7 (i)**	**NAP7 (ii)**	**NAP7 (iii)**	**NAP10**
	0.5	0.55	0.46	0.51	0.58	0.60	0.58	0.59	0.60	0.42	0.56
SEM[c]		*0.02*	*0.00*	*0.01*	*0.01*	*0.01*	*0.02*	*0.02*	*0.02*	*0.02*	*0.01*
	1	0.54	0.48	0.58	0.65	0.71	0.59	0.57	0.62	0.38	0.67
SEM		*0.01*	*0.03*	*0.01*	*0.02*	*0.01*	*0.03*	*0.02*	*0.02*	*0.03*	*0.02*
	2	0.72	0.77	0.96	0.79	0.74	0.80	0.62	0.68	0.55	0.85
SEM		*0.01*	*0.09*	*0.04*	*0.02*	*0.01*	*0.03*	*0.00*	*0.03*	*0.02*	*0.02*
	3	1.02	1.03	0.91	0.82	0.83	0.90	0.66	0.76	0.88	0.94
SEM		*0.03*	*0.01*	*0.02*	*0.02*	*0.04*	*0.05*	*0.04*	*0.04*	*0.01*	*0.05*
	4	1.05	1.04	0.99	0.93	0.93	1.01	0.65	0.73	0.97	0.95
SEM		*0.03*	*0.02*	*0.04*	*0.01*	*0.01*	*0.02*	*0.00*	*0.06*	*0.03*	*0.04*
	5	1.05	0.99	0.95	0.94	0.90	0.98	0.70	0.85	1.03	0.92
SEM		*0.02*	*0.03*	*0.01*	*0.01*	*0.01*	*0.01*	*0.01*	*0.01*	*0.03*	*0.01*

[a]7-UDCA = C7-sulfated UDCA, [b]PFGE = Pulsed-field gel electrophoresis

[c]Bottom row of each group displays the standard error of the mean (SEM) for the mean listed in the cell above.

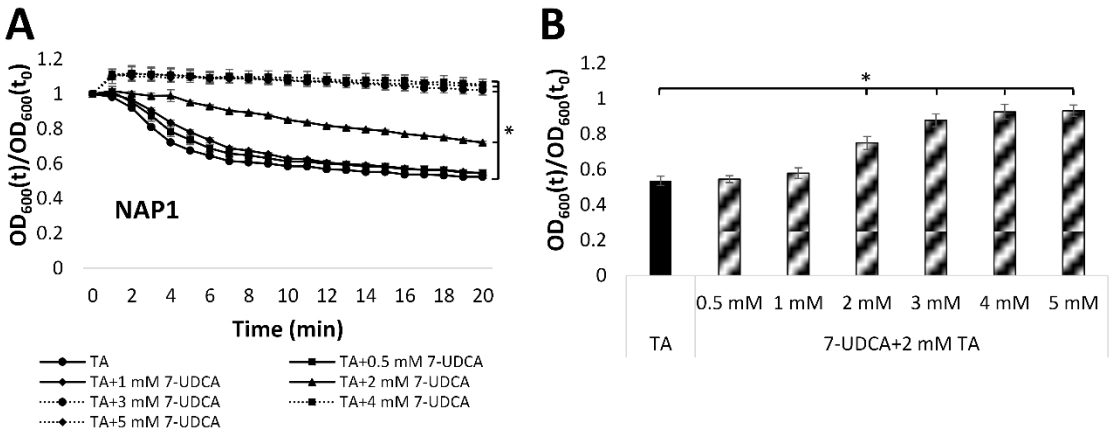


Figure 5.1 – 7-SUDCA inhibits *C. difficile* germination. A) Relative OD_{600} of NAP1 spores exposed to 0.5 mM (■), 1 mM (♦), 2 mM (▲), 3 mM (dashed line; ●), 4 mM (dashed line; ■), or 5 mM (dashed line; ♦) C7-sulfated UDCA in the presence of 2 mM TA vs. 2 mM TA alone (●). B) Mean relative OD_{600} of spores from 10 isolates after 20 min exposure to 0.5, 1, 2, 3, 4, or 5 mM C7-sulfated UDCA in the presence of 2 mM TA vs. 2 mM TA alone. $OD_{600}(t)/OD_{600}(t_0)$ = OD_{600} normalized to initial OD_{600} (relative OD_{600}); * = $p<0.01$. TA: taurocholate; 7-UDCA: C7-sulfated UDCA. Data represent mean ± SEM.

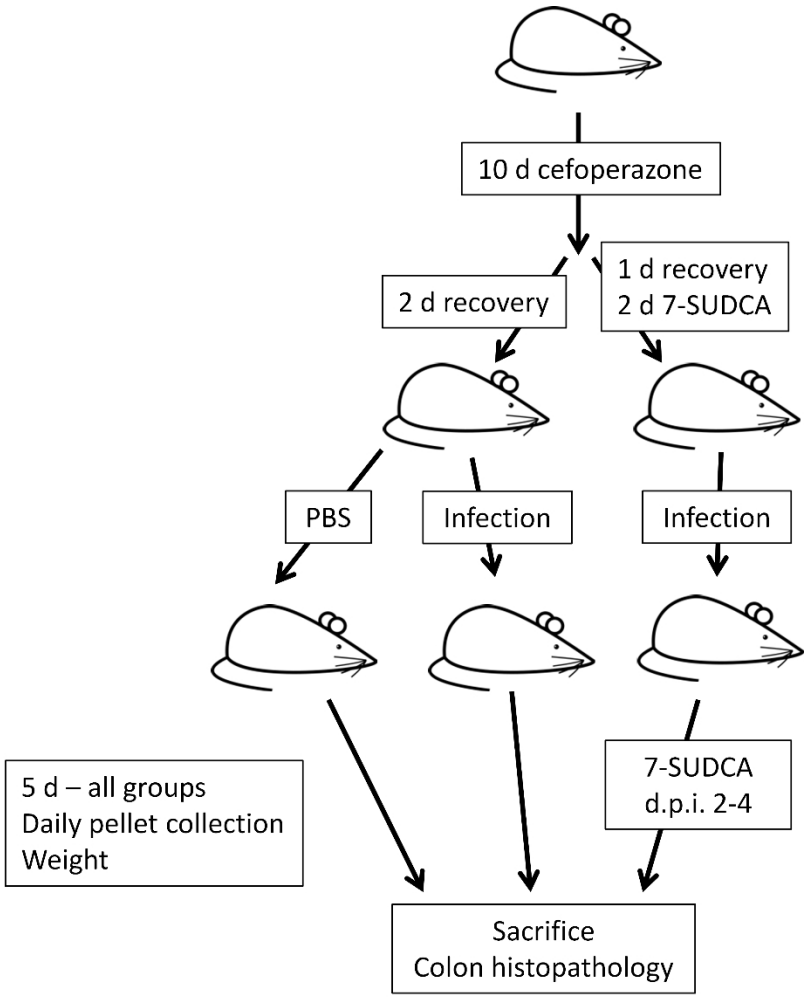


Figure 5.2 – Experimental design of mouse model. Mice were given cefoperazone (0.5 mg/mL) in drinking water for 10 days, then allowed to recover for 2 days prior to infection. One group of mice (n=2) was given 37.5 mg 7-SUDCA via oral gavage one day prior to infection and day of infection. This group and a control group (n=4) then received 2 x 10^5 *C. difficile* spores via gavage; an additional control group (n=4) received PBS alone via gavage. Following infection, fecal pellets were collected daily for *C. difficile* counts and all mice were weighed. The first group continued to receive 37.5 mg 7-SUDCA via oral gavage at 2, 3, and 4 days post-infection. After 5 days, mice were sacrificed and colons preserved for histopathology. 7-SUDCA: C7-sulfated ursodeoxycholic acid; PBS: phosphate buffered saline; d.p.i.: days post-infection.

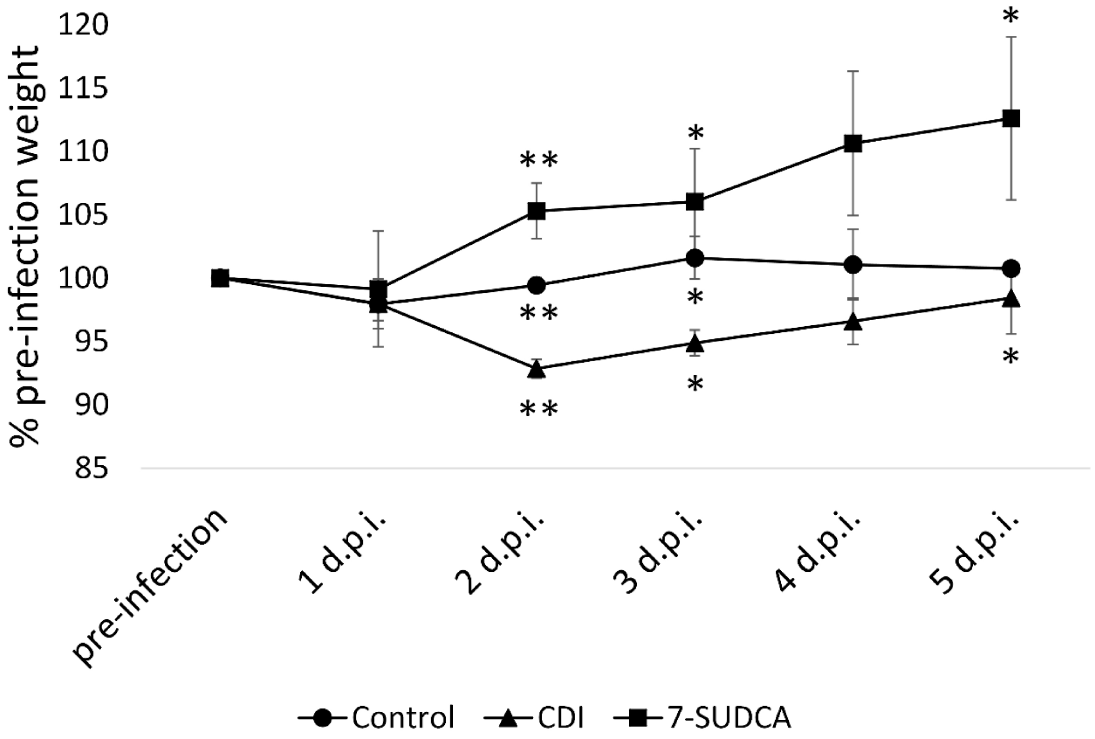


Figure 5.3 – *C. difficile*-infected mice lose more weight than control or 7-SUDCA-treated mice. Weight of control, uninfected mice (●, n=4), *C. difficile*-infected mice (▲, n=4), and *C. difficile*-infected mice treated with 7-SUDCA (■, n=2) following infection, normalized to pre-infection weight. * = $p<0.05$; ** = $p<0.01$. Error bars represent SEM. CDI: *Clostridium difficile*-infected; 7-SUDCA: C7-sulfated ursodeoxycholic acid; d.p.i.: days post-infection.

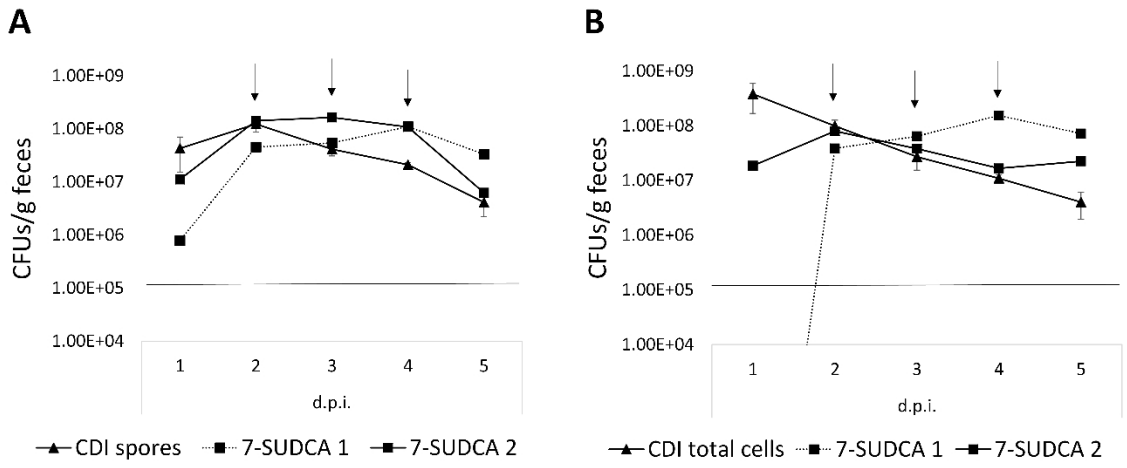


Figure 5.4 – 7-SUDCA delays appearance of *C. difficile* in feces. A) *C. difficile* CFUs from spores present in feces from control infected mice (▲, n=4) and in each infected mouse treated with 7-SUDCA (■). B) *C. difficile* CFUs from total cells (spores and vegetative cells) present in feces from control infected mice (▲, n=4) and in each infected mouse treated with 7-SUDCA (■). Arrows indicate days on which 7-SUDCA was administered. Solid line indicates limit of detection. Error bars represent SEM. CFU: colony-forming unit; d.p.i.: days post-infection; CDI: *Clostridium difficile*-infected; 7-SUDCA: C7-sulfated ursodeoxycholic acid.

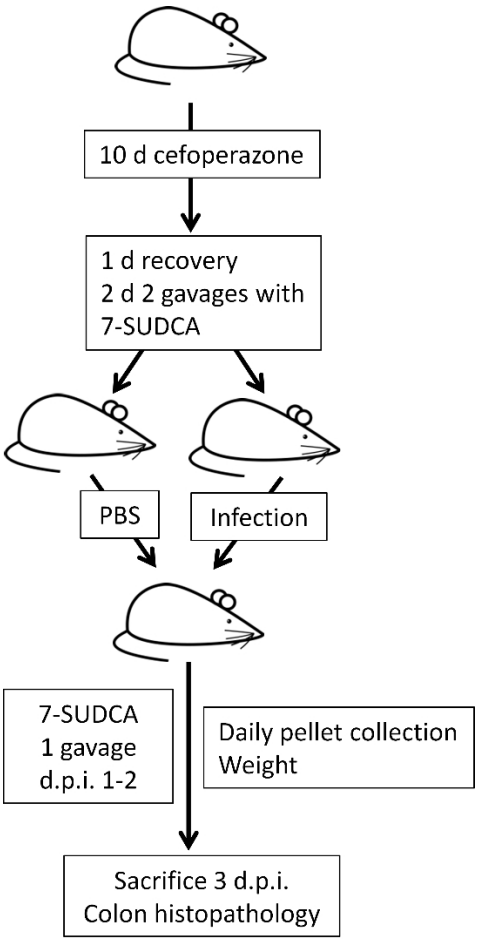


Figure 5.5 – Experimental design of second mouse experiment. Mice were given cefoperazone (0.5 mg/mL) in drinking water for 10 days, then allowed to recover for 2 days prior to infection. Both group of mice were given two gavages of 18.75 mg 7-SUDCA one day prior to infection and day of infection. One group (n=4) received 2 x 10^5 *C. difficile* spores via gavage; an additional control group (n=2) received PBS alone via gavage. Following infection, fecal pellets were collected daily for *C. difficile* counts and all mice were weighed. All mice continued to receive one gavage of 18.75 mg 7-SUDCA on 1 and 2 days following infection. After 3 days, mice were sacrificed and colons preserved for histopathology. 7-SUDCA: C7-sulfated ursodeoxycholic acid; PBS: phosphate buffered saline; d.p.i.: days post-infection.

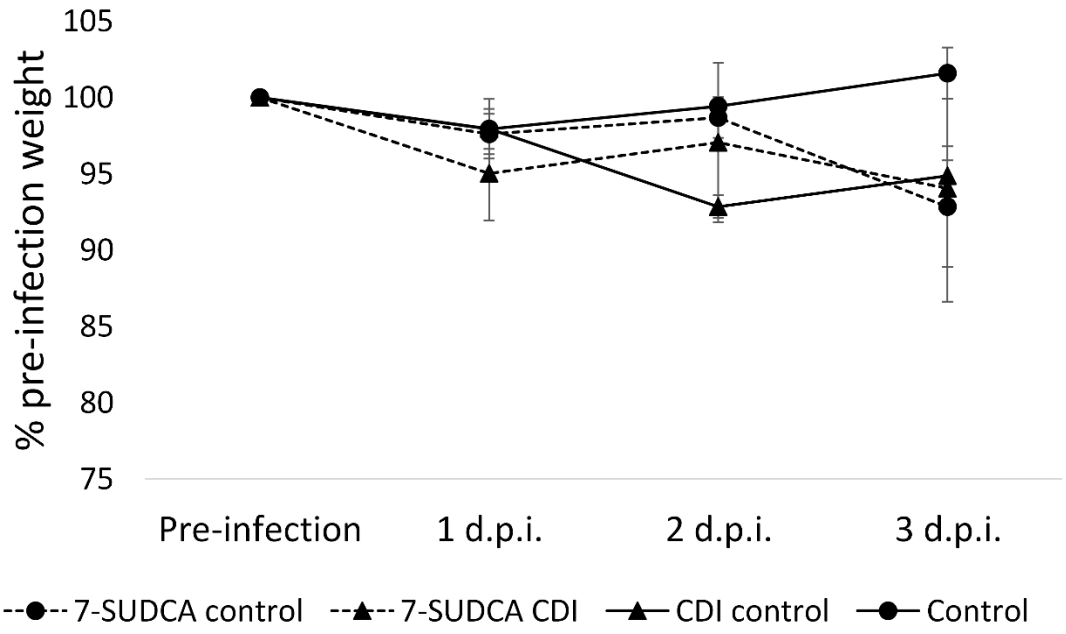


Figure 5.6 – Multiple gavages with 7-SUDCA do not inhibit weight loss associated with CDI, likely related to stress of gavage. Weight of control, uninfected mice (●, solid line, n=4), *C. difficile*-infected mice (▲, solid line, n=4), mice gavaged with 7-SUDCA but not infected (●, dashed line, n=2), and *C. difficile*-infected mice treated with 7-SUDCA (▲, dashed line, n=4 [1 d.p.i.], n=3 [2-3 d.p.i.]) following infection, normalized to pre-infection weight. Error bars represent SEM. CDI: *Clostridium difficile*-infected; 7-SUDCA: C7-sulfated ursodeoxycholic acid; d.p.i.: days post-infection.

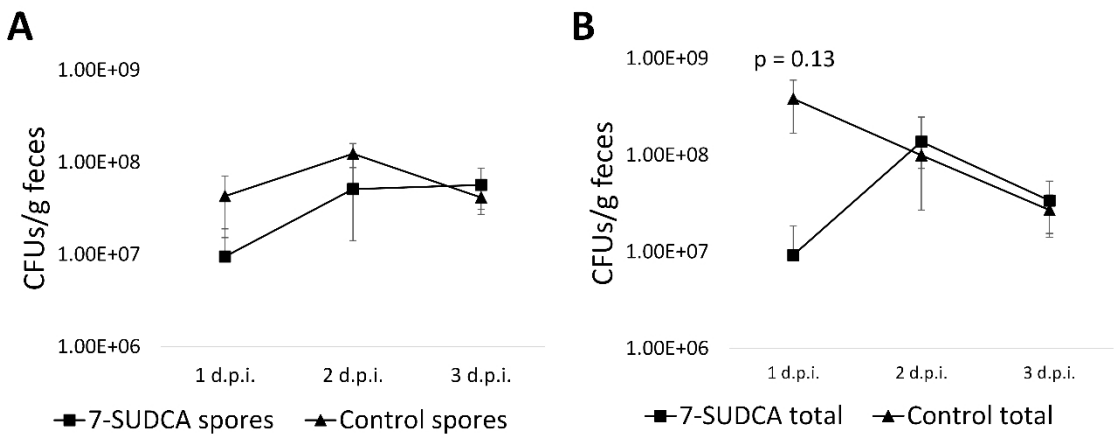


Figure 5.7 – 7-SUDCA delays appearance of *C. difficile* in feces. A) *C. difficile* CFUs from spores present in feces from control infected mice (▲, n=4) and in infected mice treated with 7-SUDCA (■, n=4 [1 d.p.i.], n=3 [2-3 d.p.i.]). B) *C. difficile* CFUs from total cells (spores and vegetative cells) present in feces from control infected mice (▲, n=4) and in each infected mouse treated with 7-SUDCA (■, n=4 [1 d.p.i.], n=3 [2-3 d.p.i.]). Error bars represent SEM. CFU: colony-forming unit; d.p.i.: days post-infection; CDI: *Clostridium difficile*-infected; 7-SUDCA: C7-sulfated ursodeoxycholic acid.

Chapter 6

Ursodeoxycholic Acid Prevents Recurrence of *Clostridium difficile* Infection-Associated Pouchitis

SUMMARY

Clostridium difficile infection (CDI) is an extremely common nosocomial infection which is often refractory to antibiotic treatment. Although fecal microbiota transplantation (FMT) is emerging as a successful therapy for the majority of patients with recurrent CDI (R-CDI), there remains a need to develop novel therapies for this disease for patients who fail the criteria for FMT or who fail to recover following the procedure. Recent work has indicated that fecal bile acid composition plays a significant role in recovery following FMT, and that ursodeoxycholic acid (UDCA), a minor human secondary bile acid clinically available as a therapeutic, can inhibit *C. difficile* germination and growth. While UDCA may be of limited therapeutic utility due to its absorption into enterohepatic circulation in the small intestine, we successfully used UDCA to control antibiotic- and FMT-refractory R-CDI pouchitis in a status post-colectomy patient. Fecal microbial community structure was tracked in this patient before and after initiation of UDCA by 16S rRNA gene sequencing and indicated that the fecal bacterial community did not recover after more than three months of UDCA therapy. These results indicate that UDCA may be an effective non-antibiotic pharmacologic agent to treat R-CDI pouchitis.

INTRODUCTION

Clostridium difficile infection (CDI) is an antibiotic-associated diarrheal disease that is currently estimated to be the most common nosocomial infection in the US (Zilberberg et al., 2008; Miller et al., 2011; Dubberke and Olsen, 2012; Magill et al., 2014). Although prior antibiotic use is considered the primary risk factor for CDI (Kelly and Lamont, 2008; Miller et al., 2011; Ananthrakishnan, 2011; Lessa et al., 2012; Khanna et al., 2012; Chitnis et al., 2013; Borody and Khoruts, 2011; Hu et al., 2009; Fashner et al., 2011), current standard therapy for this disease is additional antibiotics (Cohen et al., 2010). Unfortunately, 20-30% of CDI patients treated with antibiotics experience a recurrence of the infection once antibiotics are halted (Kelly and Lamont, 2008; Borody and Khoruts, 2011; Louie et al., 2011; Surawicz and Alexander, 2011). Once a patient has experienced a single recurrence, the chances of further recurrence may be as high as 65% or greater, and some of these patients go on to develop repeated, vicious cycles of antibiotic use and recurrence of disease (Khoruts and Sadowsky, 2011). This condition is known as recurrent CDI syndrome (R-CDI) (Borody and Khoruts, 2011).

Fortunately for patients with R-CDI, fecal microbiota transplantation (FMT) is emerging as an accepted and highly effective therapy for *C. difficile* infection. Unlike antibiotics, FMT is over 90% effective in patients with R-CDI (Hamilton et al., 2012; Kassam et al., 2013). Although the mechanisms of FMT remained obscure for more than 50 years following its first cited use in the literature (Eiseman et al., 1958), recent work has indicated that bile acid metabolism by the transplanted microbiota is a likely means of preventing further infection (Weingarden et al., 2014; Chapter 4). Notably, patients

treated with FMT undergo significant shifts in the composition of fecal bile acids after the procedure, from a bile acid pool dominated by the primary bile acids (those produced by the liver) to a pool that is predominantly secondary bile acids (produced from primary bile acids exclusively by colonic bacteria) (Weingarden et al., 2014). Many primary bile acids are known to induce *C. difficile* germination, activating previously inert spores (Wilson, 1983; Sorg and Sonenshein, 2008), while many secondary acids can prevent germination and inhibit growth of the resulting vegetative cells (Sorg and Sonenshein, 2009; Sorg and Sonenshein, 2010).

Previously, we demonstrated that the combination of bile acids present in patient feces prior to FMT induces germination and allows growth of *C. difficile*, in contrast to the combination of bile acids present after the procedure (Chapter 4). We further demonstrated that ursodeoxycholic acid (UDCA), another secondary bile acid which is not commonly found in human bile (Hofmann and Hagey, 2008) but is clinically approved for use in bile acid disorders (Lindor et al., 2009; Poupon, 2010), can also prevent both germination and growth of *C. difficile* (Chapter 4). We therefore hypothesized that UDCA could be used to prevent recurrence of CDI in human patients.

Although UDCA has been shown to be potentially inhibitory to *C. difficile* spore germination *in vitro* (Chapter 4; Sorg and Sonenshein, 2010), its efficient uptake into the enterohepatic circulation in the distal small bowel limits its intracolonic concentration and therefore its usefulness in treating R-CDI (Rodrigues et al., 1995; Hofmann and Hagey, 2008). Nonetheless, we considered UDCA as a non-antibiotic option to treat *C. difficile* infection associated with ileal pouchitis. Although *C. difficile* small bowel enteritis is rare, it is commonly associated with post-colectomy pouch dysfunction (Seril

and Shen, 2014). Similar to CDI in the colon, infection in the post-colectomy pouch can become refractory to antibiotics, and unfortunately FMT may be less effective in these patients compared to patients with normal intestinal anatomy (Hamilton et al., 2012; Borody et al., 2014; Patel et al., 2014). Therefore, there is a need for alternative treatments for patients with R-CDI pouchitis.

In this report we demonstrate the use of UDCA to control refractory *C. difficile* pouchitis in a status post-colectomy patient. The patient's isolate of *C. difficile* was found to be susceptible to UDCA, and fecal concentrations of the drug were tracked to ensure delivery to the site of infection. To assess recovery of the fecal microbiota in this patient, we also performed 16S rRNA sequencing on fecal samples before and up to three months after initiating UDCA therapy. Overall, these results support the hypothesis that UDCA can be used to prevent CDI recurrence in status post-colectomy patients.

MATERIALS AND METHODS

Isolation of C. difficile from patient feces

C. difficile from patient feces was isolated by serial dilution onto taurocholate-cycloserine-cefoxitin-fructose agar (TCCFA) followed by 48 hr anaerobic growth at 37°C (Bliss et al., 1997). Colonies were identified by PRO kit test (Remel, USA) and confirmed by sequencing the 16S rRNA gene using the primer set 515F-806R (Caporaso et al., 2012). Confirmed isolates were grown in CCFB anaerobically for 48 hr and stored in 25% glycerol at -80°C.

C. difficile spore preparation

C. difficile cells from frozen stocks were inoculated into CCFB medium and grown anaerobically at 37°C for 48 hr. Cultures were plated onto brain heart infusion with 5 g/L yeast extract and 0.1% L-cysteine (BHIS) and grown for 7 d at 37°C under anaerobic conditions. Following Sorg and Sonenshein, 2010, colonies from each plate were scraped into 1 mL of ice-cold water and incubated at 4°C overnight to release spores. A 3 mL suspension was loaded onto 10 mL of 50% (w/v) sucrose in a 15 mL conical tube and centrifuged in a swinging bucket rotor at 3200 x *g* for 20 min at 4°C. Sucrose and vegetative cells above the spore pellet were removed, and the pellet was washed 5 times in ice-cold water to remove remaining sucrose. Spores were examined under phase-contrast microscopy to determine purity; spore samples with >99% purity (<1% vegetative cells) were stored at 4°C.

C. difficile spore germination

Germination assays were done as previously described by Sorg and Sonenshein, 2010. Spores were heated to 65°C for 30 min and inoculated into BHIS, with or without UDCA and/or TA, within an anaerobic bag flushed and filled with N_2 gas. OD_{600} was measured initially ($OD6_{00}(t_0)$) and every minute for 20 min ($OD_{600}(t)$) using an EL808 Microplate Reader (Biotek Instruments, Inc., Winooski, VT). Relative OD_{600} for each time point was calculated as $OD_{600}(t)/OD_{600}(t_0)$. Experiments were performed in triplicate.

Growth of C. difficile vegetative cells

Cells from frozen stocks were inoculated into BHIS broth with 0.1% (w/v) taurocholate and incubated overnight at 37°C under anaerobic conditions. Vegetative cells were then inoculated into tubes containing BHIS, with or without UDCA, normalized to an OD_{600} of 0.005, and grown anaerobically at 37°C. Measurements of OD_{600} were collected each hour for 12 hr following inoculation, and a final OD_{600} was measured after 24 hr growth. Experiments were performed in triplicate.

Measurement of UDCA and TA in feces

Fecal samples were spiked with oleanolic acid (internal standard), suspended in 10 volumes of 50% aqueous acetonitrile, and extracted by vortexing and sonication for 10 min. The suspension was centrifuged twice at 18,000 × *g* for 10 min. The supernatant was passed through a 2 µm filter and subjected to LC-MS analysis. A 5 µl aliquot was injected into an Acquity™ UPLC system (Waters, Milford, MA) and separated by a mobile phase gradient ranging from water to 95% aqueous acetonitrile containing 0.1%

formic acid over a 10-min run. LC eluant was introduced into a Xevo-G2-S QTOF mass spectrometer (Waters) for metabolite identification and quantification. Capillary and cone voltage for electrospray ionization (ESI) were maintained at -1.5 kV and -35 V for negative-mode detection, respectively. Source temperature and desolvation temperature were 120 °C and 350 °C, respectively. Nitrogen was used as a cone (50 l/h) and desolvation gas (800 l/h), and argon was included as the collision gas. The mass spectrometer was calibrated with sodium formate solution (range *m/z* 50-1000) and monitored by the intermittent injection of the lock mass leucine enkephalin ($[M-H]^-$ = 554.2615 *m/z*) in real-time. Mass chromatograms and mass spectral data were acquired and processed by MassLynx™ software (Waters) in centroided format. UDCA and TA were identified by comparing their accurate masses, tandem MS (MS/MS) fragments, and retention times with authentic standards. Concentrations in fecal samples were determined using corresponding standard curves and QuanLynx™ software (Waters).

DNA extraction

DNA was extracted from each sample (0.25 to 0.50 g) using the PowerSoil® DNA Isolation Kit (MO BIO, Carlsbad, CA) according to the manufacturer's instructions. Samples with high water content (Bristol stool scale types 5 to 7) were centrifuged at 12,000 RPM for 3 min to pellet solids, which were used for DNA extraction. Each sample was extracted in triplicate, and each replicate was eluted in 50 µl of 10mM Tris-HCl buffer (pH 8.0) and pooled. DNA concentrations of extracted samples were measured with a QuBit® DNA quantification system (Invitrogen, Carlsbad, CA) using

QuBit high sensitivity assay reagents. All extracted DNA samples were stored at -20°C until amplification.

PCR amplification and sequencing

Fecal DNA samples were used as template for PCR amplification of the V5-V6 region of the 16S rRNA gene using a V5F/V6R Nextera primer pair (Table 6.1). Following amplification with these primers, PCR products were diluted 1:100 and amplified using Illumina indexing primers, which include flowcell adapters (Table 6.1). Pooled, size-selected PCR products from the second reaction were denatured with NaOH, diluted to 8 pM in the Illumina HT1 buffer (Illumina, San Diego, CA), spiked with 15% PhiX, and heat denatured at 96°C for 2 min immediately prior to loading onto the sequencer. A MiSeq 600 cycle v3 kit was used to sequence the samples.

Sequence processing and analysis

Reads in each pair for each sequencing run overlapped and paired ends were merged. The hamming distance (number of substitutions) was calculated for sliding overlaps of the two reads in a pair to find the best overlap (lowest hamming distance with a minimal overlap of 25 nucleotides and 98% identity). Merged sequences were binned according to barcode sequence and both barcode and amplicon primer sequences were trimmed using custom Perl scripts. Sequence data was processed and analyzed using the mothur program (Schloss et al., 2009). To ensure high quality data for analysis, sequence reads containing: ambiguous bases; homopolymers >8 bp; more than one mismatch in the primer sequence; or an average per base quality score below 35 within each 50 bp

window were removed. Sequences that only appeared once in the total set were assumed to be a result of sequencing error and removed from the analysis. Chimeric sequences were removed from the data set using the UCHIME algorithm within the mothur program (Edgar et al., 2011). A random subset of 25,189 sequences per sample were used to balance read numbers and clustered into operational taxonomic units (OTUs). Taxonomy was assigned at a cutoff value of ≥97%, using a 16S rRNA database prepared from the Ribosomal Database Project (RDP) 9, using the Bayesian method with a bootstrap algorithm (100 iterations) and a probability cutoff of 0.60 (Cole et al., 2009). Principal coordinate analysis was performed using a tree generated via Bray-Curtis method, and Shannon diversity indices were calculated using mothur.

Study approval

The study was approved by the University of Minnesota Institutional Review Board (IRB 0901M56962). The patient provided informed consent for participation in the study.

RESULTS

Clinical history of recurrent CDI pouchitis

The patient was first seen in our clinic as a 53-year old woman, 6 years status post total colectomy performed for refractory ulcerative colitis. Following her surgery, she suffered from chronic, incessant diarrhea averaging approximately 10-15 liquid bowel movements over 24 hr despite use of antidiarrheal agents, resulting in extreme fatigue and inability to sleep sufficiently. Laboratory evaluation demonstrated iron deficiency anemia and protein-losing enteropathy with serum albumin of 2.3 g/dL and moderately decreased levels of immunoglobulins G, A, and M. Endoscopic evaluation demonstrated moderate inflammation in the ileal-anal pouch, but also proximal small bowel ulcers and a stricture in the distal small bowel, which prompted a retained video capsule that was retrieved endoscopically. These findings were most consistent with Crohn's disease, and the patient was started on anti-tumor necrosis factor therapy. Although there was modest overall clinical improvement attributed primarily to intravenous iron replacement, her diarrhea was unchanged. Her stool tested positive for *C. difficile* toxin B despite absence of any recent antibiotic exposure. The patient was started on oral vancomycin and she reported marked improvement in her diarrhea, including firmer consistency of her stool unseen since her colectomy.

Unfortunately, response to vancomycin was not sustained and symptoms recurred within a week of stopping vancomycin. Additional attempts to clear the infection included two more courses of vancomycin, including a 2-month tapered regimen, and a course of fidaxomicin. Antibiotics were discontinued and FMT was performed by

endoscopic infusion of standardized fecal microbiota directly into the pouch as well as proximal small bowel. Subsequently the patient tested negative for *C. difficile* at 2 and 3 weeks post-FMT, but did not experience improvement in diarrhea. Relapse of the infection was documented at one month after FMT. Vancomycin was restarted at 125 mg four times daily, tapered down to 125 mg once daily, and maintained at that dosage for 6 months. Despite clinical improvement in the initial months, her diarrhea progressively became worse and ultimately returned to the original state despite control of *C. difficile* infection by vancomycin. Vancomycin was at this time discontinued, and the patient's stool again tested positive for *C. difficile* toxin B.

Germination and growth of C. difficile isolated from CDI pouchitis patient is inhibited by UDCA

The lack of success with antibiotic or FMT therapy in this patient prompted a trial of an alternative therapy to prevent CDI recurrence. We previously demonstrated that ursodeoxycholic acid (UDCA), a secondary bile acid available clinically, could inhibit germination and growth of *C. difficile* spores *in vitro*, suggesting it might prevent germination and growth *in vivo* and therefore prevent recurrent disease (Chapter 4). However, the utility of UDCA in patients with normal gastrointestinal anatomy is limited due to its effective absorption into enterohepatic circulation (Rodrigues et al., 1995; Hofmann and Hagey, 2008). But in this patient, who was status post-colectomy, we hypothesized that oral UDCA would reach the site of infection and prevent recurrence of CDI.

To assess whether UDCA might be used to treat this patient, we isolated *C. difficile* from a sample of the patient's feces collected during active infection and examined whether UDCA could inhibit germination of spores generated from this isolate. An established spectrophotometric assay that relies on the refractive nature of bacterial spore coats (Sorg and Sonenshein, 2008; Sorg and Sonenshein, 2009; Sorg and Sonenshein, 2010; Heeg et al., 2012) was used to examine germination of this isolate when spores were exposed to a known germinant, taurocholate (TA), versus TA in the presence of a range of concentrations of UDCA. The relative OD_{600} of spores exposed to any concentration of UDCA (0.5, 1, or 2 mM) was significantly ($p<0.01$) higher compared to spores exposed to TA alone (Fig. 6.1A), suggesting that concentrations of UDCA as low as 0.5 mM could prevent germination of spores from this patient's isolate of *C. difficile*.

To assess growth in the presence of UDCA, overnight cultures were grown with TA to induce germination, and resulting vegetative cells were inoculated (to OD_{600} = 0.005) into BHIS medium or BHIS containing 2 mM UDCA. After 24 hours, the growth of cells exposed to UDCA was significantly ($p<0.0001$) lower than cell grown in BHIS alone (Fig. 6.1B), indicating that UDCA inhibited vegetative growth of this isolate of *C. difficile*. Overall, these findings implied that UDCA could be an effective therapy to prevent CDI recurrence in this patient.

Successful treatment of recurrent CDI pouchitis with oral UDCA

Based on our *in vitro* results, the patient was prescribed oral UDCA (300 mg twice daily), which was well tolerated. After two weeks, the UDCA dose was increased

to 300 mg four times daily (20 mg/kg/day) and oral vancomycin (125 mg/day) was discontinued. The frequency of diarrhea decreased and firm bowel movements were reported. Furthermore, the stool remained negative for *C. difficile* by PCR tests for toxin B over nine months following termination of vancomycin.

The concentrations of UDCA and TA were measured in patient feces before and up to 94 d after initiation of therapy. There was a marked increase in UDCA and decrease in TA following UDCA dose increase (Fig. 6.2). Together with our *in vitro* findings, these results indicate that an oral UDCA dose of 300 mg four times daily is sufficient to suppress taurocholate-induced *C. difficile* germination at concentrations measured in the feces.

Fecal bacterial communities do not normalize following UDCA therapy

Although we successfully prevent recurrence of CDI in this patient, likely due to ongoing inhibition of *C. difficile* germination with UDCA, we wanted to assess whether oral UDCA therapy could be halted. Because R-CDI patients whose fecal microbiota is normalized via FMT rarely experience further CDI recurrences (Khoruts et al., 2010; Shahinas et al., 2012; Hamilton et al., 2013; van Nood et al., 2013; Song et al., 2013; Weingarden et al., 2014; Seekatz et al., 2014; Fuentes et al., 2014), we hypothesized that after halting vancomycin, this patient's fecal microbiota could normalize and UDCA might be stopped.

To test this hypothesis, we sequenced the bacterial 16S rRNA gene from the patient's fecal samples before and up to 94 days after initiating UDCA therapy. From a total of 1,408,244 sequences, each sample was subsampled to 25,189 sequences before

clustering into operational taxonomic units (OTUs) at a cutoff of 97% sequence similarity. OTUs were classified by bacterial phylum and family (Fig. 6.3). Although the fecal microbiome of healthy individuals is dominated by the *Bacteroidetes*, *Firmicutes*, and *Actinobacteria* phyla (Zoetendal et al., 2008; Arumugam et al., 2011), prior to beginning UDCA, this patient's fecal microbiome was predominantly *Firmicutes* and *Proteobacteria* (Fig. 6.3A), similar to what has been observed with other patients who experience recurrent CDI (Khoruts et al., 2010; Shahinas et al., 2012; Hamilton et al., 2013; van Nood et al., 2013; Song et al., 2013; Weingarden et al., 2014; Seekatz et al., 2014; Fuentes et al., 2014). Early after initiating oral UDCA, the *Fusobacteria* also became a dominant phylum, representing between 10% and 45% of OTUs up to 35 days after starting UDCA. The relative abundance of *Proteobacteria* decreased from 75% of OTUs at 7 days after therapy initiation to 25% of OTUs 80 days after starting UDCA; however, at 94 days post-initiation the relative abundance of *Proteobacteria* returned to 70% of OTUs. The *Bacteroidetes* phylum remained undetectable until the last time point, 94 days after starting UDCA, and represented only 2% of OTUs at that point. These findings suggest that the bacterial phyla present in this patient's feces remain altered compared to healthy individuals even nearly three months after stopping antibiotic therapy.

Similar to the phyla, the bacterial families present in this patient's feces also remained altered after beginning UDCA therapy (Fig. 6.3B). The majority of *Proteobacteria* in this patient's fecal communities appeared to be *Enterobacteriaceae*, as this family represented between 10 and 75% of fecal OTUs in all samples. Similarly, an increase in the *Fusobacteriaceae* family was likely responsible for the increase in the

Fusobacteria phylum following initiation of UDCA. The *Lachnospiraceae* family, within *Firmicutes,* was present during active *C. difficile* infection at a fairly high relative abundance of 35% of OTUs, but quickly became undetectable during vancomycin therapy. Although this family recovered at 35 days following initiation of UDCA (21 days after halting vancomycin), it became undetectable once more at 94 days after starting UDCA. Although *Bacteroidaceae* and *Prevotellaceae,* within *Bacteroides,* are thought to be major constituents of the human microbial flora (Arumugam et al., 2011), neither family was detectable in any sample. Similar to findings at the phylum level, these results suggest that the fecal microbiome of this patient had not recovered by 94 days after initiating UDCA.

To analyze overall fecal bacterial community structure and diversity of this patient, we compared these samples to samples collected from nine healthy individuals who participated our microbial therapeutics program (Khoruts et al., 2015). Shannon diversity indices, which represent diversity within samples (α-diversity), indicated that the α-diversity of fecal samples from this patient was lower at all time-points compared to healthy individuals (Fig. 6.4A). Principal coordinate analysis (PCoA), which is a reflection of diversity between samples (β-diversity), indicated that while fecal bacterial communities from healthy individuals were similar, communities in this patient were distinct from healthy samples, although initiation of UDCA therapy did cause a noticeable shift in community structure (Fig. 6.4B). These findings indicate that initiation of UDCA therapy did not result in normalization of either α- or β-diversity, even up to 94 days after the start of treatment.

Overall, although shifts in bacterial communities were detectable in the patient's feces after initiation of UDCA and after halting vancomycin, these changes did not reflect full recovery of the fecal microbiota even after three months on the drug. This suggests that the patient could still be at risk for CDI recurrence, were she to stop oral UDCA.

DISCUSSION

Clostridium difficile infection (CDI) is now considered the most common hospital-acquired infection in the US (Zilberberg et al., 2008; Miller et al., 2011; Dubberke and Olsen, 2012; Magill et al., 2014) and community-acquired cases are also increasingly found (Khanna et al., 2012; Lessa et al., 2014; Lessa et al., 2015). Unfortunately, many patients with CDI go on to develop recurrent CDI syndrome (R-CDI), characterized by repeated cycles of antibiotic therapy and reoccurring symptoms (Borody and Khoruts, 2011). For these patients, fecal microbiota transplantation (FMT) may be the most promising therapy, offering a far higher rate of cure than standard antibiotic treatments (Kassam et al., 2013). Despite the efficacy of FMT, however, there remains a need to develop novel therapies for this disease for individuals who fail the criteria for FMT or fail to remain infection-free following the procedure.

Although CDI is primarily a colonic disease for individuals with normal intestinal anatomy, *C. difficile* infection is also associated with 10% of diarrheal symptoms in patients with an ileal pouch reservoir that is surgically created after a total colectomy (Seril and Shen, 2014). Similar to colonic CDI, ileal pouch CDI can become recurrent and difficult to eradicate with antibiotics. Thus, there is a need for alternative approaches. FMT has been suggested for R-CDI pouchitis, but its efficacy in this situation is currently unknown. Patel and colleagues reported the use of FMT in one case (Patel et al., 2014), but the patient did not respond symptomatically and was continued on anti-CDI antibiotics for a month after the procedure, ultimately eradicating CDI. In our experience, large segment partial colectomy has been associated with initial FMT failure (Hamilton et

al., 2012) and other investigators have noted difficulty curing R-CDI with FMT following subtotal colectomy (Borody et al., 2014). Bacterial production of secondary bile acids, thought to be a critical mechanism of FMT which prevents recurrence of CDI (Weingarden et al., 2014; Chapter 4), may be intrinsically limited in these patients, although this deserves systematic study. However, the absence of the colon also allows for greater delivery of UDCA to the site of infection, enhancing its usefulness in the treatment of CDI-associated pouchitis. This idea is supported by the case report presented here of a patient who failed to clear CDI in the ileal pouch following multiple courses of antibiotics and FMT, but was successfully treated with oral UDCA.

In addition to tracking clinical outcomes, we also tracked the fecal microbiota of this patient, in part to determine whether normalization of the fecal microbiota occurred during treatment with UDCA. Patients with R-CDI, including the patient presented here, frequently have fecal microbiota that are highly altered from healthy individuals, including predominance of the *Proteobacteria* phylum and *Enterobacteriaceae* family (Khoruts et al., 2010; Shahinas et al., 2012; Hamilton et al., 2013; van Nood et al., 2013; Song et al., 2013; Weingarden et al., 2014; Seekatz et al., 2014; Fuentes et al., 2014), compared to dominance by the *Bacteroidetes*, *Firmicutes*, and *Actinobacteria* phyla in normal feces (Zoetendal et al., 2008; Arumugam et al., 2011). Although the relative abundance of *Proteobacteria* in the feces of this patient decreased after vancomycin was halted, it remained a common phylum in her fecal communities, and in fact increased in relative abundance at our latest time point (94 d post-initiation of UDCA). Furthermore, while *Firmicutes* were abundant in every fecal sample, the *Bacteroidetes* only became detectable at 94 d after starting UDCA, where they remained a relatively minor

component of the fecal microbiota. Finally, the α-diversity of the patient's fecal communities remained low compared to the healthy individuals, and β-diversity analyses indicate that community composition remained distinct from that of healthy individuals even nearly three months after cessation of vancomycin. Overall, these findings indicate that the patient's fecal bacterial community did not normalize over the time points we monitored.

It is likely that the fecal microbiota of this patient remained distinct from that of healthy individuals either due to a lingering effect of antibiotic therapy or due to the patient's altered intestinal anatomy. Even in individuals without underlying pathology, one or two antibiotic courses can drastically alter fecal microbial communities for 4-10 weeks, and can cause lasting changes in overall composition as well as to individual taxa for up to 10 months (Dethlefsen et al., 2008; Dethlefsen and Relman, 2011). The microbiota of the ileal pouch is also often distinct from normal colon. One study in a small cohort found that three of four patients had mucosal microbial communities in the ileal pouch which were distinct from healthy colonic mucosal communities (Young et al., 2013). Interestingly, the patient whose communities were most similar to colonic communities remained pouchitis-free for the duration of the study, unlike the other three patients. Another study found that patients with underlying ulcerative colitis (UC) have distinct pouch microbiota even compared to the microbiota of patients who are status post-colectomy for familial adenomatous polyposis (FAP): compared to FAP patients, UC patients have increased *Proteobacteria* (up to 90% of the microbiota) and decreased *Bacteroidetes* in the ileal pouch (Zella et al., 2011), similar to the findings in our patient.

Overall, our findings suggest that UDCA is a promising therapeutic for patients with R-CDI of the ileal pouch. However, our findings as well as previous findings indicate that the microbiota of the pouch may not normalize compared to the microbiota of the healthy colon. As a result, patients may need to remain on UDCA chronically. However, because our results represent the findings in one individual, further systematic trials are now needed to evaluate UDCA as a potential non-antibiotic treatment of CDI-associated pouchitis, especially in cases of recurrent disease. Furthermore, tracking of the microbiota in these patients, as well as additional sampling points in the patient presented here, may be indicated in order to determine whether the microbiota ultimately normalize.

Table 6.1 – Primers used in 16S sequencing.

Primer	Sequence
V5F Nextera	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGRGGATTAGATACCC
V6R Nextera	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCGACRRCCATGCANCACCT
Forward Index Primer	AATGATACGGCGACCACCGAGATCTACAC[i5][a]TCGTCGGCAGCGTC
Reverse Index Primer	CAAGCAGAAGACGGCATACGAGA[i7][a]GTCTCGTGGGCTCGG

[a][i5] and [i7] refer to index sequence codes for barcoding used by Illumina.

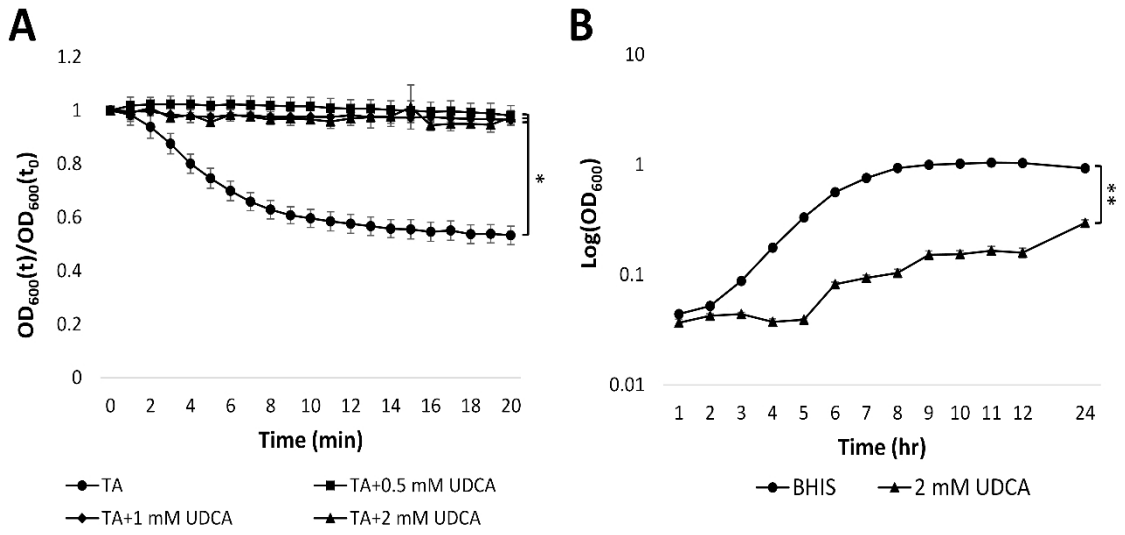


Figure 6.1 – UDCA inhibits germination and growth of *C. difficile* isolated from patient stool. A) Relative OD_{600} of spores isolated from patient exposed to 0.5 mM (■), 1 mM (♦), or 2 mM (▲) UDCA in the presence of 2 mM TA vs. 2 mM TA alone (●). $OD_{600}(t)/OD_{600}(t_0)$ = OD_{600} normalized to initial OD_{600} (relative OD_{600}). B) Growth of vegetative cells from *C. difficile* isolate in BHIS alone (●) or BHIS with 2 mM UDCA (▲). * = $p<0.01$, ** = $p<0.0001$. TA: taurocholate; UDCA: ursodeoxycholic acid. Data represent mean ± SEM.

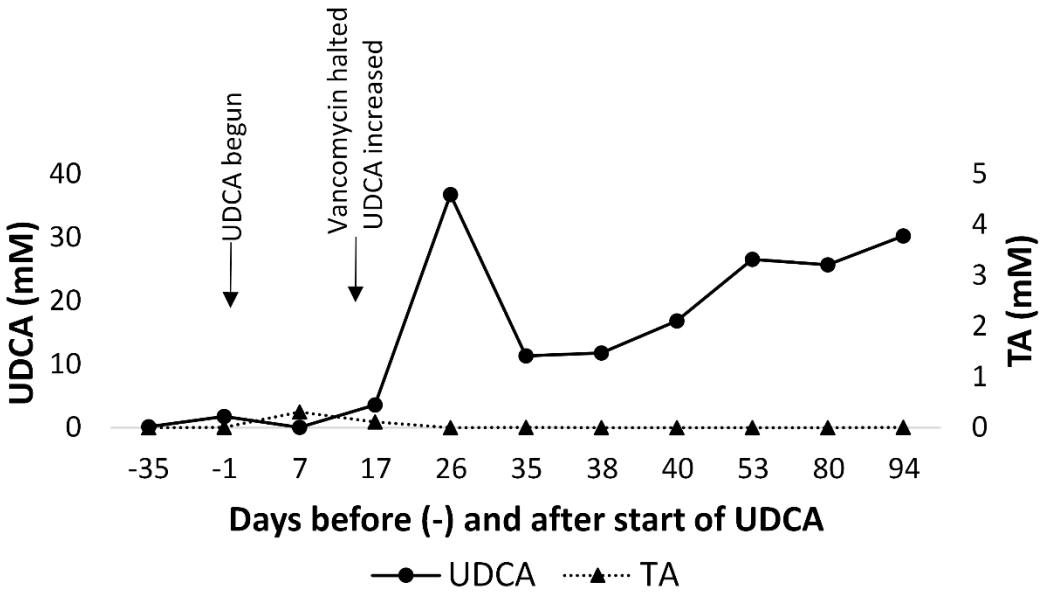


Figure 6.2 – UDCA is detectable in patient feces. Concentration of UDCA (●) and taurocholate (▲) in patient feces before and after initiation of UDCA therapy. Timing of significant clinical events is noted. TA: taurocholate; UDCA: ursodeoxycholic acid.

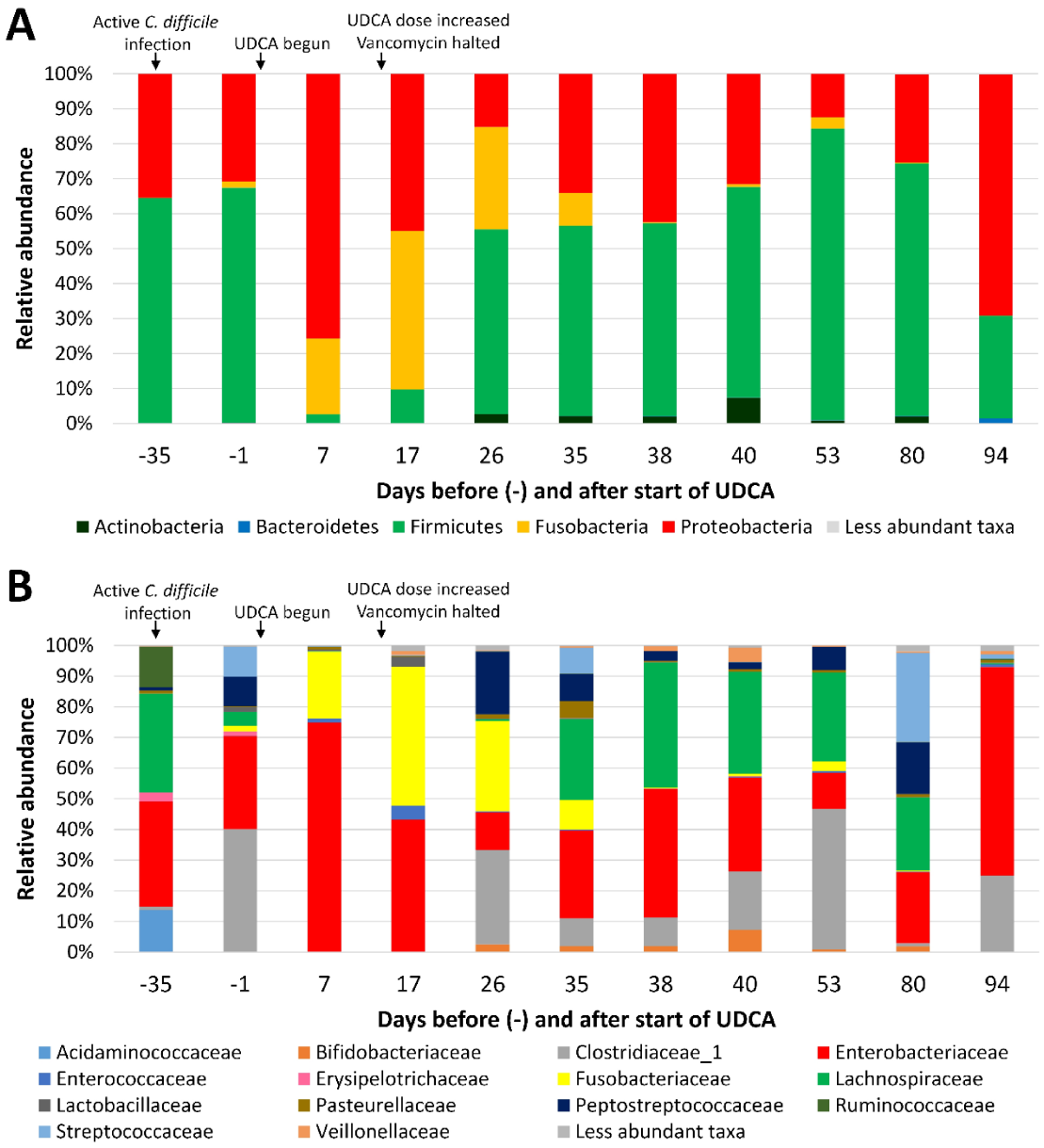


Figure 6.3 – Fecal bacterial communities are altered after initiating UDCA. A) Relative abundance of sequences from bacterial phyla in patient feces before and after start of UDCA therapy. See legend below for list of phyla. B) Relative abundance of sequences from bacterial families in patient feces before and after start of UDCA therapy. See legend below for list of families. Significant clinical events are noted above.

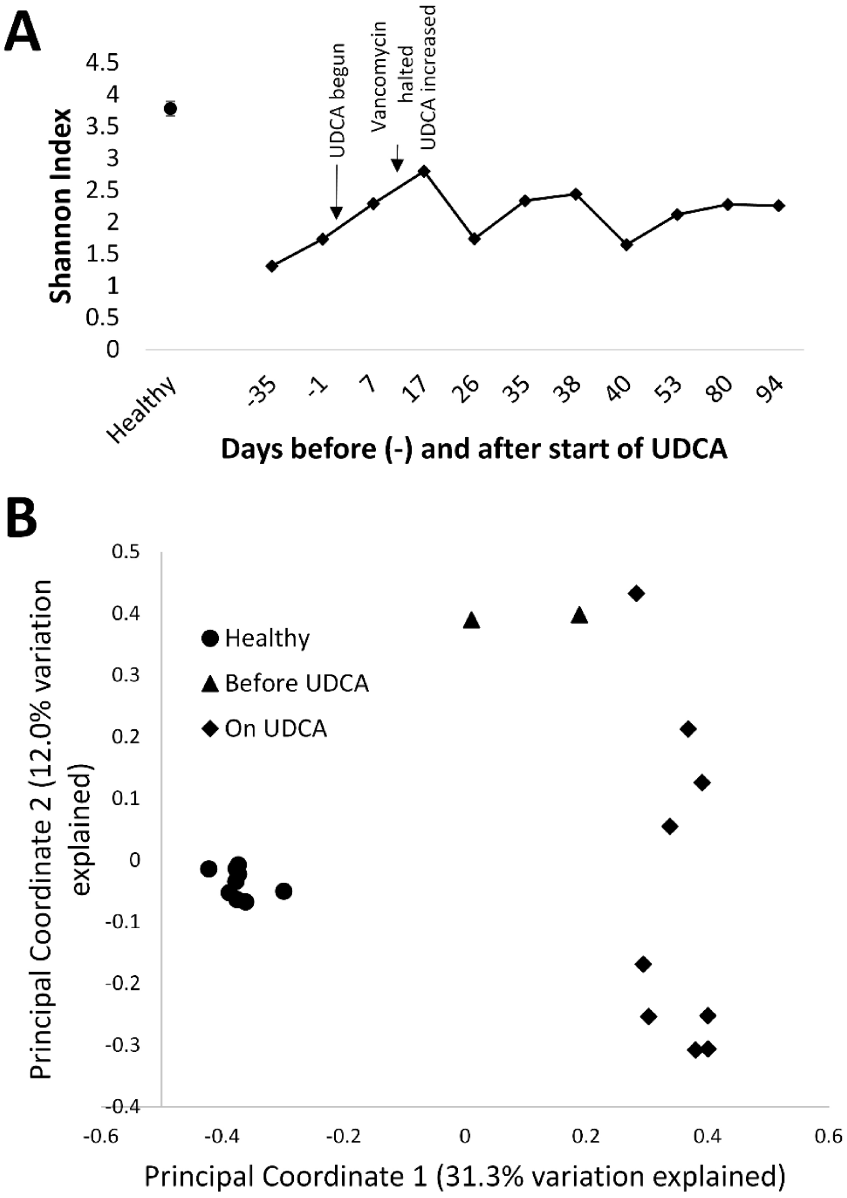


Figure 6.4 – α- and β-diversity of patient fecal samples do not normalize following UDCA therapy. A) Shannon diversity indices of healthy (●) and patient (♦) fecal samples before and after initiating UDCA therapy. Significant clinical events are noted above. Healthy data represent mean ± SEM. B) Principal coordinate analysis of healthy (●) and patient fecal communities before (▲) and after (♦) initiating UDCA therapy.

Chapter 7

Stabilization of Distal Gut Bacterial Composition Following Sequential Fecal Microbiota Transplantation to Treat Severe *Clostridium difficile* Infection[‡]

[‡] Partially reprinted from *Journal of Clinical Gastroenterology*. Alexa R. Weingarden, Matthew J. Hamilton, Michael J. Sadowsky, and Alexander Khoruts. "Resolution of severe *Clostridium difficile* infection following sequential fecal microbiota transplantation." **Originally published in *Journal of Clinical Gastroenterology*. 2013. 47(8):735-737.**

SUMMARY

Severe *Clostridium difficile* infection (CDI) is associated with high mortality even with surgical intervention. Fecal microbiota transplantation (FMT) is a potential alternative treatment approach, but one that has not yet been mechanistically investigated. The primary aim of this study was to measure changes in the bacterial composition of fecal microbiota following FMT in treatment of severe CDI that is refractory to standard antibiotic therapy. This is a clinical case series of four patients treated by FMT, using frozen fecal microbiota, for severe CDI. Bacterial composition of distal gut microbiota was characterized before and after FMT using high-throughput 16S rRNA gene sequencing of fecal samples in three of four patients. FMT resulted in initial clinical improvement in all cases. However, the subsequent course was characterized by recurrence of CDI. This was associated with instability of the bacterial composition of fecal microbiota, specifically increasing abundance of *Enterobacteriaceae* and decreasing abundance of *Lachnospiraceae* and *Bacteroidaceae* family members. Optimal donor engraftment with stabilization of the fecal microbiota composition and clearing of CDI was achieved by a second FMT following an intervening course of antibiotics. FMT is a promising alternative to surgical treatment of severe CDI. However, a single FMT may not achieve sustained protection against recurrence of CDI in this clinical situation. Our experience suggests that the double FMT protocol described here achieves sustained recovery from severe CDI.

INTRODUCTION

Over the past two decades *Clostridium difficile* infection (CDI) has steadily risen in both incidence and severity in North America and Europe, and is currently the most common infection associated with healthcare (Miller et al., 2011). This epidemic correlates with emergence of new, hypervirulent strains of *C. difficile,* characterized by greater potential for exotoxin production and sporulation efficiency, which likely contribute to greater disease severity and recurrence rates (Merrigan et al., 2010). About 3-10% of patients with CDI progress to a severe, complicated, or fulminant state, which can be highly lethal. Severe CDI can progress rapidly to multisystem organ failure, and early surgical intervention, typically subtotal colectomy, can be lifesaving (Markelov et al., 2011). However, even surgical treatment of CDI in practice is associated with postoperative mortality rates of 35-80% (Dallal et al., 2002; Koss et al., 2006; Wysowski, 2006; Lamontagne et al., 2007).

Fecal microbiota transplantation (FMT) is an alternative to standard antibiotic and surgical therapy, which is used mainly for multiply recurrent CDI (Bakken et al., 2011; Gough et al., 2011; Borody and Khoruts, 2011). This procedure aims to repair the fundamental defect that allows the establishment of CDI: suppression and disruption of the microbial communities normally inhabiting the distal gut by antibiotics. However, only a handful of case reports exist on the use of FMT in severe or fulminant CDI (You et al., 2008; Brandt et al., 2011; Gallegos-Orozco et al., 2012; Trubiano et al., 2012). The earliest published experience on successful clinical use of FMT by Eiseman and colleagues in 1958 focused on severe disease associated with pseudomembranous colitis

(Eiseman et al., 1958). The approach has not gained traction in medical practice, likely due to a number of practical constraints which includes a very narrow window of intervention in addition to other concerns such as aesthetic challenges, no standardization, and lack of reimbursement (Hamilton et al., 2012). Extremely limited clinical experience and lack of investigations into the mechanism of microbiota restoration undoubtedly also contribute to hesitation in using this approach to treat severe CDI.

In the course of the past several years we have established a clinical program of FMT for multiply recurrent CDI at the University of Minnesota. Most serious barriers associated with use of FMT have been overcome by developing a standardized protocol of preparation of fecal microbiota material that is nearly odorless and can be stored in a frozen state until needed for the procedure (Hamilton et al., 2012). In the course of this experience we were called to perform FMT on four patients with severe CDI, who were poorly responsive to antibiotic therapy and had prohibitive surgical risk. Each case was associated with a unique clinical narrative, united by the overall degree of complexity associated with a critical illness in the context of multiple medical co-morbidities. We measured the clinical response to FMT and characterized the composition of the fecal microbiota in these patients before and after the procedure.

MATERIALS AND METHODS

Patients

The four patient cases represent a consecutive experience in treating severe *C. difficile* infection with FMT at the University of Minnesota from March 2011 to February 2012. Surgical teams were involved in all cases, and the decision to perform FMT was done following establishment of consensus among all the participating medical and surgical services. The procedures were done after obtaining informed consent and the patients or their families also consented to participate in the study of bacterial composition of their feces before and after FMT. The Institutional Review Board at the University of Minnesota approved prospective collection of clinical and fecal microbial composition data (project approval date: October 2, 2009), while recognizing this experience does not constitute a formal clinical trial testing efficacy of FMT in comparison to any other therapeutic options.

Case Histories

Patient 1. 66 year old woman was admitted to the University Hospital with six day history of progressive diarrhea following recent treatment of sinusitis with two courses of amoxicillin. Her past medical history was most notable for metastatic ovarian carcinoma diagnosed and surgically debulked seven years earlier. She had received multiple courses of different chemotherapy in the intervening years. Her fourth cycle of paclitaxel was administered four weeks prior to admission. The patient reported severe diarrhea, continuous through day and night, along with chills, fatigue, and anorexia. Notable

findings on physical examinations included tender lower abdomen, which was otherwise soft and flat. Computerized tomography (CT) scan of her abdomen demonstrated diffuse colonic wall thickening without pneumatosis, pericolonic fat stranding, and small amout of ascites. She had slightly increased white blood cell (WBC) count of 12.6 x 10^9/L and albumin of 3.4 g/dL. Her stool studies were positive for *C. difficile* toxin B by PCR. The patient was initially started on oral metronidazole, but changed to oral vancomycin in the next two days as she failed to show any clinical improvement. On day 2 of hospitalization her abdomen was noted to be markedly distended, tympanitic, and diffusely tender. She developed fevers of 39.5°C, fluid bolus refractory hypotension with blood pressure of 70/40 mmHg and metabolic acidosis with bicarbonate level of 12. Antibiotic management at this time included oral rectal vancomycin and intravenous metronidazole. The patient received an infusion of intravenous immunoglobulin. On day 3 of hospitalization she was transferred to the medical intensive care unit where she was placed on ventilator and vasopressor support. Surgical consultant concluded that subtotal colectomy was the surgical procedure of choice, but would carry high risk of mortality, and cited metastatic ovarian carcinoma and extremely low albumin of 1.3 to be complicating factors. The consultant recommended to consider FMT prior to committing to subtotal colectomy.

Antibiotics were discontinued at mid-day of hospitalization day 3 and two liters of polyethylene glycol electrolyte solution (GoLYTELY) were administered via nasogastric tube. FMT was performed via colonoscopy in the late evening of hospitalization day 3 using freshly prepared fecal material from a pre-screened volunteer donor. The colonoscope was gently advanced to the hepatic flexure and severe pseudomembranous

colitis was noted (Fig. 7.1A). Within several hours of the procedure there was hemodynamic improvement with lessening vasopressor support and defervescence. WBC count decreased from peak of 24.3 x 10^9/L to 10.8 x 10^9/L within 24 hours (Fig. 7.1B). Over the next several days her clinical recovery was described as "remarkable." However, on day 6 after the procedure clear signs of clinical deterioration were noted with increasing diarrhea, abdominal pain, and distended abdomen. Unfortunately, the option to perform a second FMT was not available due to out of town absence of the performing endoscopist. The patient was taken to surgical subtotal colectomy. She was able to recover over the next six weeks and continues to be clinically stable.

Patient 2. 74 year old man was admitted to an outside hospital with abdominal pain, watery diarrhea, nausea, and vomiting, about two and a half weeks following treatment of foot cellulitis with clindamycin. He had numerous medical problems, including severe emphysema with oxygen dependence, atherosclerotic heart disease, history of aortic aneurism repair, recurrent aspiration pneumonias, including an episode a year ago requiring mechanical ventilation, narcotic dependent back pain, and legal blindness due to glaucoma. He was afebrile and hemodynamically stable, although his WBC count was 20.4 x 10^9/L and albumin was 3.2 g/dL. He was admitted and started empirically on oral metronidazole. His stool studies were positive for *C. difficile* toxin B by PCR, and also grew out vancomycin resistant enterococcus. Over the next three days he showed little clinical improvement and started having fevers up to 39.5°C. Intravenous ertapenem was administered for suspected systemic infection and metronidazole was changed to intravenous route of administration. The patient was reported increasing abdominal pains

and required fluid resuscitation for hypotensive episodes. CT scan of the abdomen showed thickening of the colon wall from cecum to sigmoid colon. There was a moderate amount of ascites. The patient was transferred to the medical intensive care unit, started on oral vancomycin, and surgical consultation was obtained for consideration of colectomy. The surgeon determined that his multiple medical co-morbidities greatly increased the likely mortality of subtotal colectomy and recommended stopping intravenous antibiotics and transferring the patient to the University hospital for consideration of FMT.

On re-evaluation at the University the patient appeared very tired. His systolic blood pressure was in the 90s mmHg. Abdomen was distended, mildly tender diffusely, and bowel sounds were absent. WBC count was 40.0 x 10^9/L, albumin was 2.0 g/dL, and C-reactive protein (CRP) was 208 mg/L; these laboratory indicators were monitored daily through the remainder of his hospital course (Fig. 7.2A). Vancomycin was held for 24 hours during which the patient received several liters of GoLYTELY via nasogastric tube. FMT was done the next day via colonoscopy, during which pseudomembranous colitis was seen to involve the entire colon (Fig. 7.2A). The patient appeared clinically improved the following day and reported complete resolution of abdominal pain. On the second day following the procedure he had a solid bowel movement. However, his bowel movement was loose on day 3 after the procedure and he noted return of fatigue. He had several more loose bowel movements on day 4, and the worsened symptoms correlated with increased CRP and WBC counts (Fig. 7.2A). On day 5 his stool was positive for *C. difficile* toxin B by PCR. The patient was restarted on oral vancomycin and was discharged to a rehabilitation facility. A second FMT was performed two weeks

following discharge at which time signs of pseudomembranous colitis were absent (Fig. 2B). The patient had no relapse of *C. difficile* infection in the subsequent one year despite two episodes of aspiration pneumonia.

Patient 3. 68 year old woman was admitted to the University hospital with hematochezia, abdominal pain, and increasing nausea five weeks following Nissen fundoplication surgery. She had a complex past medical history complicated by a cognitive disorder and a narcotic dependent chronic pain syndrome related to a motor vehicle accident five years previously, heavy tobacco dependence, hypertension, and polyneuropathy, among a number of other medical and surgical items. She was tachycardic on admission with heart rate of 131 beats per minute and blood pressure of 182/92 mmHg. She was febrile with a temperature of 38.1°C. Abdomen was diffusely tender, but not distended. WBC count was 24.1 x 10^9/L and albumin was 4.1 g/dL. CT of the abdomen demonstrated marked colonic wall thickening extending from rectosigmoid to the mid-transverse colon with mild surrounding fat stranding and small amount of ascites. Extensive atherosclerosis was noted in the abdominal aorta and iliac arteries, although the proximal celiac axis and the superior mesenteric artery were noted to be patent. Despite these findings, the primary suspected diagnosis was ischemic colitis. The patient was started on intravenous piperacillin/tazobactam and metronidazole. There was little clinical improvement over the next three days as the patient required increasing amounts of narcotics for pain control and continued to have tachycardia and low grade fevers. *C. difficile* infection was considered, but fecal samples failed to be collected during this time. CT angiogram was performed demonstrating patent inferior mesenteric artery proximally and in its major

branches. A flexible sigmoidoscopy performed on day 4 of hospitalization due to persistent rectal bleeding described dark, edematous mucosa with confluent large pseudomembranes. *C. difficile* toxin B was found to be positive on day 5 of hospitalization by PCR. The primary diagnosis was changed to that of severe *C. difficile* colitis, and non-CDI antibiotics were discontinued. Oral vancomycin was added to the intravenous metronidazole. Nevertheless, the patient continued to have diarrhea and complain of severe abdominal pain, concordant with diffuse abdominal tenderness seen on physical examination. This CDI antibiotic therapy continued for another five days without significant change in the clinical status. However, on day 11 she developed worsening abdominal distension with cessation of stools. Abdominal X-ray showed dilatation of the transverse colon to 6 cm. Antibiotics were discontinued, and the patient received GoLYTELY in preparation for FMT; the primary service also opted to simultaneously administer intravenous immunoglobulin at 1 g/kg. On hospitalization day 12 the patient was noted to be more lethargic than the previous day.

The FMT was performed via colonoscopy on hospitalization day 12, noting pseudomembranous colitis most severe in the transverse colon as well as a 6 mm polyp in the rectum. There was little clinical improvement seen on day 13 as the patient became increasingly somnolent and spiked a fever of 38.6°C. Fidaxomicin was initiated despite resolution of clinical dilatation on hospitalization day 14, followed by abdominal CT scan that demonstrated decreased thickening of the colon and decreased amounts of ascites. On hospitalization day 15 her abdominal exam was markedly improved compared to all preceding days. However, her mental status worsened further. She was extremely lethargic, and was no longer able to follow simple commands or identify her husband.

Analysis of cerebrospinal fluid obtained by lumbar puncture showed moderate pleocytosis with 11 WBC/μL, protein 48 mg/dL, and glucose of 54 mg/dL. Stool testing for *C. difficile* was negative. Neurological assessment was that the patient experienced aseptic meningitis caused by intravenous immunoglobulin administered to the patient shortly before the FMT. She was also discovered to have cystitis with >100,000 Gram negative rods cultured from the urine and received a three day course of trimethoprim/sulfamethoxazole starting on hospitalization day 19. The neurological status gradually improved over five days following the lumbar puncture. Dificid was continued until day 24 and a repeat FMT was performed via colonoscopy on day 25. There were no signs of pseudomembranous colitis during this examination although some regenerative changes were still present in the transverse colon mucosa. The adenomatous polyp noted previously was resected and the patient was transferred to a rehabilitation unit on day 27. The patient continued to undergo rehabilitation for the next six months and her bowel function remained normal.

Patient 4. 83 year old woman was admitted with diarrhea and abdominal pain four days after completion of a 10 day course of amoxicillin/clavulanate, following hospitalization for gallstone induced pancreatitis and ascending cholangitis. The patient had refused cholecystectomy and was not interested in ever having surgery for any reason. Her past medical history was notable for type II diabetes, major depression, and at least mild dementia. She was tachycardic but afebrile. Abdomen was non-distended. CT scan of the abdomen demonstrated marked mural thickening with surrounding fat stranding involving the rectum, sigmoid colon, and descending colon. WBC count on admission

was 17.2 x 10^9/L and albumin was 3.2 g/dL. In addition, she was found to have a urinary tract infection for which she was started on intravenous levofloxacin along with oral vancomycin. Stool studies were positive for *C. difficile* toxin B by PCR, and the patient was started on intravenous metronidazole. Over the next three days the patient developed increasing abdominal pain and distension, and increasing mental confusion. Abdominal X-ray demonstrated dilatation of the sigmoid colon to 6 cm. Levofloxacin was discontinued on day 3 of the hospitalization and the CDI antibiotics were stopped on day 4. WBC count at this time was 19.7 x 10^9/L and CRP level was 71.3 mg/L. The patient was administered GoLYTELY prep and underwent FMT on day 5 by colonoscopy, during which she was noted to have pseudomembranous colitis greatest on the left side along with diverticulosis. Clinical improvement was noted the day after the procedure with lesser abdominal pain and distension. WBC count came down to 8.9 x 10^9/L over two days, at which time CRP level was 24.8 mg/L. The patient remained off antibiotics for three days after FMT, after which she was started on fidaxomicin with plans to repeat her FMT after completion of the 10 day course of this antibiotic. The patient was discharged to a nursing home. However, she refused to have the second FMT and returned to the hospital approximately six weeks after finishing fidaxomicin with abdominal pain and diarrhea. Abdominal CT scan showed massive dilatation of the colon. Stool studies were positive for *C. difficile* toxin B. The patient refused further care, including antibiotics, and elected hospice care for comfort.

Transplantation of fecal microbiota

All patients were on antibiotics prior to FMT. Systemic antibiotics, including metronidazole, were discontinued for at least 48 h prior to the procedure. Vancomycin, which is poorly absorbed, was discontinued 12-24 h prior to the procedure, and the patients were administered 2-3 liters of polyethylene glycol electrolyte solution (GoLYTELY®) via NG tube or orally. FMT was performed using a standardized preparation of concentrated fecal microbiota as previously described (Hamilton et al., 2012). Patient 1 received the preparation the day it was processed. For Patients 2-4, the bacterial preparation was frozen in 10% (v/v) glycerol and stored frozen at -80ºC until used. The same donor was used for all FMT procedures (Hamilton et al., 2012).

FMT was performed via colonoscopy by an experienced endoscopist with the assumption of higher perforation risk in the setting of severe CDI. The colonoscope was advanced gently to the farthest point possible, while maintaining minimal loop formation. Carbon dioxide was used for insufflation during the colonoscopy procedures. The donor fecal microbiota material was injected into the proximal colon via the biopsy channel of the colonoscope.

Sample collection

Fecal samples were collected by hospital staff from patients prior to FMT and for several days following FMT, and stored at -80°C within 24 h of production until DNA extraction. An unprocessed portion of each donor sample was also collected at the time of donation and stored immediately at -80°C.

DNA extraction

DNA was extracted from each patient and donor sample (0.25 to 0.50 g) with the PowerSoil® DNA Isolation Kit (MO BIO, Carlsbad, CA) according to the manufacturer's instructions. Samples with high water content (Bristol stool scale types 5-7) were centrifuged at 12,000 RPM for 3 min to pellet solids, which were used for DNA extraction. Each sample was extracted in triplicate, and each replicate was eluted in 50 µl of 10mM Tris-HCl buffer (pH 8.0) and pooled. DNA concentrations of extracted samples were measured with a QuBit® DNA quantification system (Invitrogen, Carlsbad, CA) using QuBit high sensitivity assay reagents. All extracted DNA samples were stored at -20°C until amplification.

PCR amplification

Fecal DNA samples (25 ng) were used as template for PCR amplification of the V6 region of the 16S rDNA gene. Primer sets (Table 7.1) were designed with a 6 bp ID tag on the 5' end of either the forward or reverse primer, which was specific to each fecal DNA sample and allowed for multiplexed sequencing. Triplicate reactions were purified using the Qiaquick® PCR Purification Kit (Qiagen, Valencia, CA), eluted in 50 µl of 10 mM Tris-Cl buffer, pH 8.0, and pooled. DNA concentrations were measured using the QuBit® DNA quantification system and high sensitivity assay reagents. Samples were stored frozen at -20^{o} C until pooled for sequencing.

DNA sequencing

Equimolar aliquots of each product from Patient 2 and donor (10 samples) were pooled and equimolar aliquots of each product from Patients 3 and 4 and donors (15 samples)

were pooled to give two samples of ~1 µg of DNA in 100 µL total volume. Final pooled DNA concentration was measured by using the Quant-IT™ PicoGreen quantitation system (Invitrogen, Carlsbad, CA). Amplicon size analysis was done using an Agilent DNA 1000 chip and a 2100 BioAnalyzer (Agilent, Santa Clara, CA). Library preparation and sequencing, done using Illumina technology, was performed at the University of Minnesota BioMedical Genomics Center. Paired-end sequences from Patient 2 and donor samples were generated on the HiSeq 2000 sequencer (100 nt read length), with 1-3 pooled samples per lane following Illumina multiplexing protocols. Paired-end sequences from Patients 3 and 4 and donor samples were generated on the MiSeq Personal Sequencer (150 nt read length). Reads in each pair for both sequencing runs overlapped and paired ends were merged. The hamming distance (number of substitutions) was calculated for sliding overlaps of the two reads in a pair to find the best overlap (lowest hamming distance with a minimal overlap of 25 nucleotides and 98% identity). Merged sequences were binned according to barcode sequence and barcode and amplicon primer sequences were trimmed using custom Perl scripts.

Sequence processing and analysis

Sequence data was processed and analyzed using the mothur program (Schloss et al., 2009). To ensure high quality data for analysis, sequence reads containing: ambiguous bases; homopolymers >7 bp; more than one mismatch in the primer sequence; or an average per base quality score below 25 within each 15 bp window were removed. Sequences that only appeared once in the total set were assumed to be a result of sequencing error and removed from the analysis. Chimeric sequences were removed from

the data set using the UCHIME algorithm within the mothur program (Edgar et al., 2011). A random subset of 152,259 sequences from each sample for Patient 2 and donor and 128,381 sequences from each sample for Patients 3 and 4 and their respective donors were used to balance read numbers and clustered into operational taxonomic units (OTUs). Taxonomy was assigned at a cutoff value of >90%, using the Ribosomal Database Project (RDP) 7 16S rRNA database using the Bayesian method with a bootstrap algorithm (100 iterations) and a probability cutoff of 0.60 (Cole et al., 2009). Samples were clustered using the Fast UniFrac algorithm to generate trees and principle coordinate analysis (PCoA) plots (Hamady et al., 2010). The UniFrac algorithm was run using the Fast Unifrac program available at www.bmf2.colorado.edu/fastunifrac/.

RESULTS

Clinical Summary

Each patient in this series had a unique clinical narrative, and individual histories are provided in Methods and Materials. These four patients developed severe CDI refractory to standard antibiotic therapy. Subtotal colectomy was considered in all patients, but was felt to be prohibitively risky or was refused by the patient (Patient 4). FMT was performed emergently in Patient 1 (Fig. 7.1A) using freshly prepared fecal material from a pre-screened donor in the medical intensive care unit (MICU). The other patients received their FMT using previously frozen material. All patients had a prompt positive clinical response to the procedure. This was especially dramatic in Patient 1 where hemodynamic improvement was evident with lessening vasopressor support, defervescence, and precipitous fall in white blood cell (WBC) count to near normal levels over 24 h (Fig. 7.1B). The improvement, however, was not sustained. Symptoms and signs of CDI returned in Patients 1 and 2 within 3-6 days (Fig. 7.1B and Fig. 7.2A). Patient 1 underwent subtotal colectomy. Patient 2 was restarted on vancomycin on day 5 after FMT and continued on vancomycin for 2 wk as an outpatient, which was followed by a second FMT. Going forward, after the experience with these two patients, we recommended re-initiation of antibiotics after a holding period of several days following the first FMT, with the plan to repeat the FMT on an outpatient basis after completing the antibiotic course. This plan was executed on Patient 3, who was started on fidaxomicin on day 2 following her first FMT, and had the second FMT done on day 14. Patient 4 was also started on fidaxomicin on day 3 after her FMT, but refused to undergo the second

FMT. She ultimately succumbed to fulminant CDI, and elected comfort care in a hospice program.

Fecal Microbiota

Analysis of bacterial composition in fecal samples. The composition of fecal microbiota in patients before and after each FMT, as well as the donor, was examined by sequencing the V6 region of the 16S rDNA gene. A total of 13,563,547 sequences were generated, and 8,510,597 sequences remained after quality filtering and chimera removal. Sequences were subsampled to obtain the same number for each patient sample, clustered into operational taxonomic units (OTUs) at 90% sequence identity, and classified using the Ribosomal Database Project (RDP) 7 (Cole et al., 2009). The most abundant 100 OTUs contributed to more than 80% of the sequences in each sample, suggesting that any additional OTUs obtained by further sequencing would not greatly affect overall sample composition.

Decreases in Proteobacteria are associated with recovery and stability of the fecal microbiome of severe CDI patients treated with FMT. The relative abundance of each bacterial phylum in fecal samples was assessed in Patients 2, 3, and 4. In Patient 2, the sample taken before the first FMT (on Day 0) had a composition similar to the donor sample, with a fecal community dominated by *Firmicutes* and *Bacteroidetes*, although there was a higher relative proportion (20%) of Proteobacteria versus the donor sample (~5%, Fig. 7.3A). One day following the initial FMT, the fecal community of Patient 2 remained dominated by *Firmicutes* and *Bacteroidetes*, though the abundance of

Proteobacteria increased. However, on the second day following FMT (Day +2) through just before the second FMT (Day +22/Day 0), the fecal bacterial community was dominated by *Proteobacteria*, which comprised over 70-90% of OTUs in each sample. Concomitantly, the abundance of *Firmicutes* and *Bacteroidetes* decreased dramatically. These changes in the fecal microbiome on Day +2 preceded the return of the patient's symptoms on Day +3, and the patient becoming *C. difficile* toxin-positive on Day +5. In contrast, following the second FMT (on Day +23/Day +1), the fecal microbiome in this patient more closely resembled the donor sample, with decreases in *Proteobacteria* and increases in *Bacteroidetes* and *Firmicutes*, as well as the appearance of the *Verrucomicrobia*. These changes persisted the next day (Day +24/Day +2). The patient developed aspiration pneumonia within a day of collecting this fecal sample and was started on antibiotics, however despite this, he did not experience recurrent *C. difficile* infection.

The fecal microbiota of Patient 3 prior to the first FMT was markedly altered compared to the donor (Fig. 7.3B). Fecal samples taken one day prior to, and the day of, FMT (Day -1 and Day 0) were dominated by *Proteobacteria*, which represented over 90% of the OTUs. In contrast, *Firmicutes* and particularly Bacteroidetes were present only in low abundance. However, on day +1 after the first FMT and over the course of the next 9 days (Days +3, +6, and +9), the relative abundance of *Proteobacteria* decreased, with a concomitant increase in the relative abundance of *Bacteroidetes* and *Verrucomicrobia*. In contrast, the relative abundance of *Firmicutes* did not markedly change, remaining ≤ 10% of the OTUs from these samples. However, this group returned following the second FMT and remained relatively abundant (~25%) 15 d later (Day

+28/Day +15), although the abundance of Proteobacteria remained high compared to the donor sample.

Similar to Patient 2, a fecal sample from Patient 4 prior to FMT (Day 0), as well as a flushed colonoscopy sample obtained prior to delivery of the donor's microbiota (Day 0 during FMT), had a microbial community similar to the donor, with a relatively high abundance of *Bacteroidetes* and *Firmicutes* and few *Proteobacteria* (Fig. 7.3C). Although the *Bacteroidetes* decreased in this patient 3 days post-procedure (Days +1, +2, and +3), these samples did not become dominated by *Proteobacteria* as was seen in Patients 2 and 3. However, 10 days post-FMT (Day +10), over 90% of OTUs represented *Proteobacteria*, while the *Firmicutes* dramatically decreased and the *Bacteriodetes* virtually disappeared. These changes in the fecal microbiome coincided with the patient's use of fidaxomicin. Unfortunately, this patient elected not to receive a second FMT, and, unlike Patients 2 and 3, experienced CDI recurrence.

***Lachnospiraceae* and *Bacteroidaceae* are associated with stability of the fecal microbiome in severe CDI patients**. We also compared the relative abundance of bacterial families in each fecal sample. In Patient 2, the intestinal microbiota prior to the first FMT (Day 0) was dominated by *Enterobacteriaceae* and *Enterococcaceae*, with low abundance of *Lachnospiraceae* compared to the donor sample (Fig. 7.4A). Although the first sample following FMT (Day +1) was characterized by a return of the *Lachnospiraceae*, with a decrease in the relative abundance of *Enterococcaeae*, the fecal microbiome by Day +2 was still dominated by *Enterobacteriaceae*, with very low abundance of the *Lachnospiraceae*, *Porphyromonadaceae*, and *Bacteroidaceae*. This

pattern persisted until immediately prior to the second FMT (Day +22/Day 0). In contrast, following the second FMT (Day +23/Day +1, Day +24/Day +2), the fecal microbiota of Patient 2 showed an increase in the relative abundance of *Lachnospiraceae*, *Porphyromonadaceae*, and *Bacteroidaceae*, with a dramatic decrease in the abundance of *Enterobacteriaceae*. Several other groups found in higher abundance in the donor sample also increased following the second FMT, including the *Ruminococcaceae* and *Rikenellaceae*.

The fecal microbiome of Patient 3 prior to the first FMT was heavily dominated by *Enterobacteriaceae*, which comprised >95% of OTUs (Fig. 7.4B). Between the first and second FMT (Days +1 through +9), the microbiota slowly shifted, with eventual increases in groups seen in higher abundance in the donor sample, including *Bacteroidaceae*, *Porphyromonadaceae*, and *Rikenellaceae*. Only after the second FMT (Day +28/Day +15), however, did the *Lachnospiraceae* appear. Interestingly, the fecal microbiome of Patient 3 after the second FMT still displayed a high relative abundance of *Proteobacteria* (Fig. 7.3B), but these were primarily unclassifiable families, rather than the *Enterobacteriaceae*.

The relative abundance of bacterial families in the fecal microbiome of Patient 4 revealed differences not seen at the phylum level (Fig. 7.4C). The intestinal microbiota of Patient 4 before the procedure (Day 0, Day 0 Tx) had slightly increased abundances of *Enterobacteriaceae* and *Porphyromonadaceae*, and lower abundances of *Bacteroidaceae* and *Lachnospiraceae*, relative to the donor. Following FMT (Days +1, +2, and +3), there was an initial increase in *Lachnospiraceae* and *Sutterellaceae*, with decreases in the relative abundance of *Enterobacteriaceae* and *Porphyromonadaceae*, although the

Bacteroidaceae also appeared to decrease. Interestingly, however, these changes reversed by 10 days post-FMT (Day +10), with loss of *Lachnospiraceae, Bacteroidaceae,* and *Porphyromonadaceae*, and replacement by *Enterobacteriaceae* and *Sutterellaceae*. This occurred concurrently with the initiation of fidaxomicin treatment 9 days following the procedure.

Fecal microbiota transplantation alters the overall fecal bacterial community in severe CDI patients. The Fast UniFrac distance metric, followed by PCoA based on weighted and normalized UniFrac distances, was used to analyze changes in the community structure of fecal microbiota in patients following FMT (Hamady et al., 2010). After the first FMT, there was an observable shift in the fecal bacterial community of Patient 2, away from the pre-treatment sample (Day 0) and towards the donor sample on the first day following FMT (Day +1, Fig. 7.5A). However, concurrent with the changes observed in the relative abundance of bacterial taxa, from Days +2 to +22, the bacterial community became distinct from both the initial sample and donor sample. Following the second FMT, the fecal microbial community in Patient 2 continued to change and was closer to, though still distinct from, the donor's microbiota.

In contrast, the bacterial communities in samples from Patient 3 were initially quite distinct from that of the donor's (Day -1 and Day 0, Fig. 7.5B). Following the initial FMT, the community shifted closer to the donor along the first principal coordinate, but appeared to change along the second principal coordinate away from both the donor and the patient's pre-FMT samples. Two weeks after the second FMT, the fecal microbial community of Patient 3 was not shifted substantially closer to the donor communities

than before the second procedure, though it remained distinct from the pre-FMT community (Day +28/Day +15).

Changes in the fecal bacterial community in Patient 4 followed a different course than either Patients 2 or 3. Interestingly, the community was relatively similar in the donor sample and pre-FMT samples from Patient 4 (Day 0 and Day 0 Tx, Fig. 7.5C). FMT in this patient did result in a substantial shift in the microbial community, but a shift away from both pre-procedure and donor samples (Day +1, +2, and +3). However, concomitant with changes in the relative abundance of phyla and families, a dramatic shift occurred on Day +10, with the development of a community highly distinct from any previous sample from this patient or the donor sample.

DISCUSSION

The case histories described in this report illustrate the common medical complexity of patients with severe CDI. In addition, review of these histories can identify early missteps taken in their care, perhaps reflecting a historical perception of CDI as a relatively mild and manageable complication of antibiotic therapy. However, the recent emergence of more toxigenic strains of *C. difficile* and rising mortality associated with CDI make this assumption no longer tenable (McDonald et al., 2005; Kelly and Lamont, 2008). Severe CDI is a potentially lethal disease, and even the best surgical treatment is associated with high mortality (Dallal et al., 2002; Koss et al., 2006; Wysowski 2006; Lamontagne et al., 2007). FMT has the potential to correct the underlying problem associated with CDI and should be investigated as a much less invasive alternative to surgery. We observed short-term clinical improvement in our four patients following FMT. The improvement was particularly impressive in Patient 1 where close monitoring in the MICU allowed visualization of hemodynamic stabilization within hours of the procedure. Although FMT appeared sufficient to stabilize the patients for several days, CDI ultimately returned in Patients 1 and 2. After these two experiences we continued the double FMT protocol in subsequent cases, where the two procedures are separated by a two-week course of antibiotics. We did not wait for recurrence of CDI in Patient 3, and she did well with this protocol. Patient 4 did not complete the recommended treatment course and ultimately succumbed to fulminant *C. difficile* colitis associated with megacolon.

It is likely that medical complexities of these patients are reflected in the complicated changes to their fecal microbiota following FMT. Of the three patients whose fecal microbiota were sequenced, only one (Patient 3) displayed dominance by members of the phylum *Proteobacteria* prior to FMT, a trait found in recurrent CDI patients and other diseases associated with microbial dysbiosis (Swidsinksi et al., 2005; Frank et al., 2007; Chang et al., 2008; Khoruts et al., 2010; Wang et al., 2012). In contrast, the fecal communities of Patients 2 and 4 had high relative abundances of *Bacteroidetes* and *Firmicutes* prior to FMT, a pattern commonly found in healthy individuals, despite these patients' critical clinical conditions (Turnbaugh et al., 2007). These findings mimic previous work comparing the fecal microbiota of patients with initial versus recurrent CDI, suggesting that patients with severe CDI do not always display the phylum-level dysbiosis seen in recurrent CDI (Chang et al., 2008).

Despite variation in bacterial phyla in the feces of these patients, there are consistent patterns in family-level abundances. In fecal samples from all three patients, a relatively high abundance of *Enterobacteriaceae* and a relatively low abundance of *Lachnospiraceae* compared to donor samples were found prior to FMT. In Patients 2 and 3, who received a second FMT, the *Enterobacteriaceae* decreased and the *Lachnospiraceae* increased for more than one day only following the second FMT. In Patient 3, this is particularly unexpected because she was treated between procedures with fidaxomicin, an antibiotic shown to spare the *Lachnospiraceae* (Tannock et al., 2010; Louie et al., 2012). While similar changes are observable in Patient 4 following the first FMT, these changes were reversed 10 days following the procedure. In both Patients 2 and 4, the dominance of *Enterobacteriaceae* and the decrease in *Lachnospiraceae*

occurred prior to CDI recurrence. Taken together, these findings suggest that the stability of the fecal microbiome in patients following FMT for severe CDI may be associated with the presence of members of the family *Lachnospiraceae*. In contrast, an unstable community structure of the fecal microbiome, perhaps indicating risk of CDI recurrence, may be associated with the dominance of *Enterobacteriaceae*. These findings reflect previous data, which suggest that the *Enterobacteriaceae* are associated with diseases such as IBD and recurrent CDI (Swidsinski et al., 2005; Frank et al., 2007; Chang et al., 2008; Khoruts et al., 2010; Wang et al., 2012). In addition, previous results have indicated that the *Lachnospiraceae* are associated with the mucosal surface of healthy individuals and may positively influence gut health, via regulation of the immune system and production of molecules which act as fuel for colonic epithelial cells (Pryde et al., 2002; Hamer et al., 2008; Atarashi et al., 2011; Nava and Stappenbeck, 2011). Furthermore, in a recent mouse model, *Lachnospiraceae* strains prevented *C. difficile* colonization, highlighting the importance of this group in prophylaxis and possibly treatment of this disease (Reeves et al., 2012). The results presented here are consistent with a potential role of *Lachnospiraceae* in patient recovery and microbiome stability in severe CDI as well.

Recurrence of CDI following initial FMT in these patients differs from our extensive experience treating multiply recurrent CDI in outpatients by FMT, where one infusion of donor fecal microbiota is sufficient in approximately 92% of cases (Hamilton et al., 2012). One possible explanation for this difference is that these hospitalized patients returned immediately to their beds, likely contaminated with *C. difficile*, and succumbed to reinfection before the donor microbiota could fully occupy their niches in

the recipient's colon. In fact, we are currently testing the hypothesis that heavy household contamination may be a major reason for FMT failures in treating multiply recurrent CDI. The instability of the fecal microbiome following a single FMT in Patients 2-4 found in this work, as indicated by shifts in microbial community structure in PCoA, suggests that colonization niches may indeed be unoccupied following a single FMT, allowing for CDI recurrence. Interestingly, Eiseman and colleagues used multiple daily enemas in treating their critically ill patients with pseudomembranous colitis, a strategy that may have facilitated stabilization of the colonic microbial community during the first several days of treatment (Eiseman et al., 1958). In patients with severe CDI, therefore, although a single FMT may assist in amelioration of life-threatening colitis, two FMT procedures appear to be superior for stable engraftment of donor microbiota and full clinical recovery.

In summary, our experience in this case series adds to the limited literature on the use of FMT for treatment of severe CDI refractory to antibiotic therapy. Notably, a single FMT only provided temporary clinical stabilization and was not sufficient to achieve stability of the colonic microbial community. However, this treatment was sufficient to allow another course of antibiotics and later administration of a second FMT on an outpatient basis for longer-term results. The standardized, frozen preparation we described earlier for recurrent CDI decreases practical concerns with this approach, which typically must be implemented on an urgent basis (Hamilton et al., 2012). Such a preparation of fecal microbiota should also simplify execution of randomized clinical trials, which should be performed in treatment of severe CDI to compare against surgical outcomes.

Table 7.1 - Primer sequences used in this study.

Primer Name	Sequence (5' - 3')
Forward Primer 1	CNACGCGAAGAACCTTANC
Forward Primer 2	CAACGCGAAAAACCTTACC
Forward Primer 3	CAACGCGCAGAACCTTACC
Forward Primer 4	ATACGCGARGAACCTTACC
Forward Primer 5	CTAACCGANGAACCTYACC
Reverse Primer 1	[6bp ID tag]CGACAGCCATGCANCACCT
Reverse Primer 2	[6bp ID tag]CGACAACCATGCANCACCT
Reverse Primer 3	[6bp ID tag]CGACGGCCATGCANCACCT
Reverse Primer 4	[6bp ID tag]CGACGACCATGCANCACCT

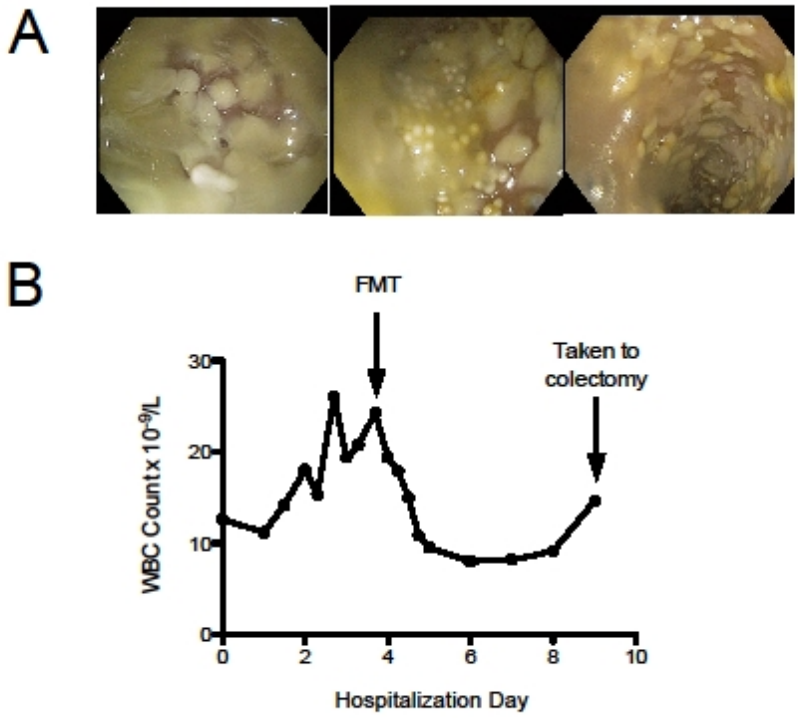


Figure 7.1 - Clinical response of Patient 1 to FMT. A) Pseudomembranous colitis was noted in images (left to right) of the sigmoid, transverse, and hepatic flexure colon. B) White blood cell count was tracked daily during hospitalization; times of fecal microbiota transplantation and colectomy are noted. Legend: FMT = fecal microbiota transplantation, WBC = white blood cell.

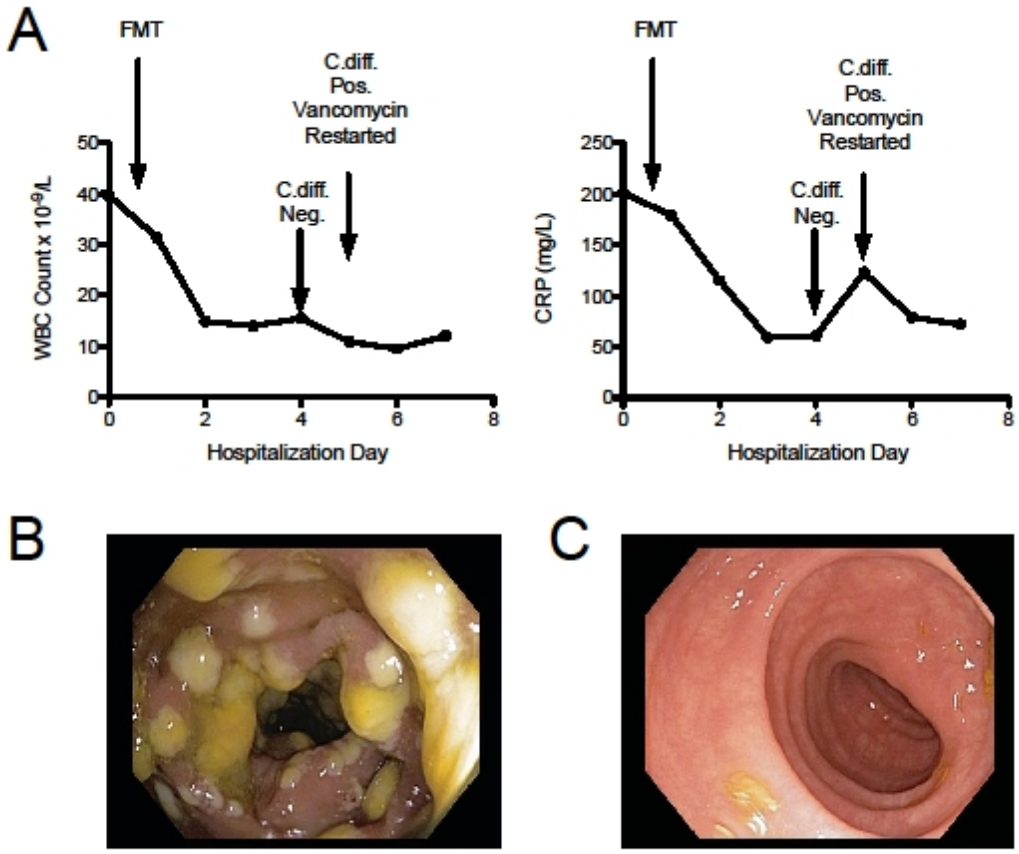


Figure 7.2 - Clinical response of Patient 2 to FMT. A) White blood cell count (left) and C-reactive protein (right) were tracked daily during hospitalization. B) Pseudomembranous colitis was observed in the sigmoid colon prior to first FMT. C) Resolution of colitis in the same site prior to second FMT. Legend: FMT = fecal microbiota transplantation; WBC = white blood cell, CRP = C-reactive protein; C. diff. Neg. = stool samples were negative for *C. difficile* toxin B by PCR; and C. diff Pos. = stool samples were positive for *C. difficile* toxin B by PCR.

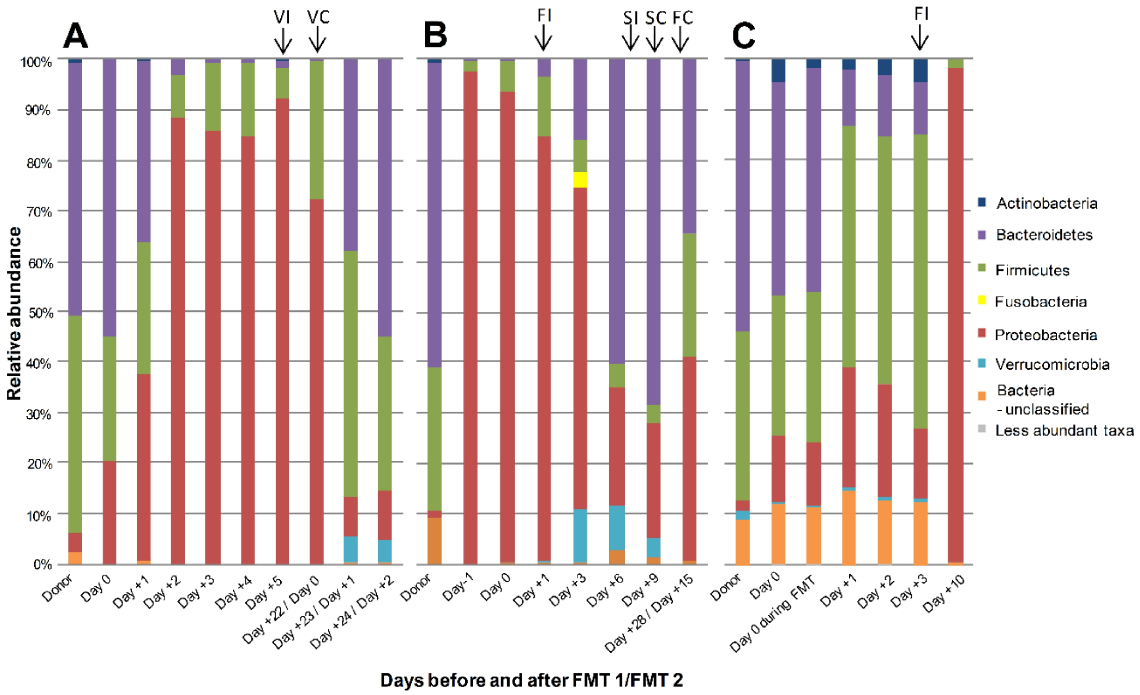


Figure 7.3 – Fecal bacterial phyla undergo change following each FMT. Relative abundances of bacterial phyla in fecal samples across treatment course in Patient 2 (A), Patient 3 (B), and Patient 4 (C), as well as donor samples. Colors correspond to phyla (see key on figure). Legend: Day 0 during FMT = sample taken from colonoscopy flush prior to FMT; and less abundant taxa = less than 0.5% of total sequences. Numbers before slash indicate days after FMT 1; numbers after slash indicate days after FMT 2. Arrows indicate initiation or cessation of antibiotics: VI = vancomycin initiation, VC = vancomycin cessation, FI = fidaxomicin initiation, FC = fidaxomicin cessation, SI = trimethoprim-sulfamethoxazole (TMP-SMX) initiation, SC = TMP-SMX cessation (see Materials and Methods).

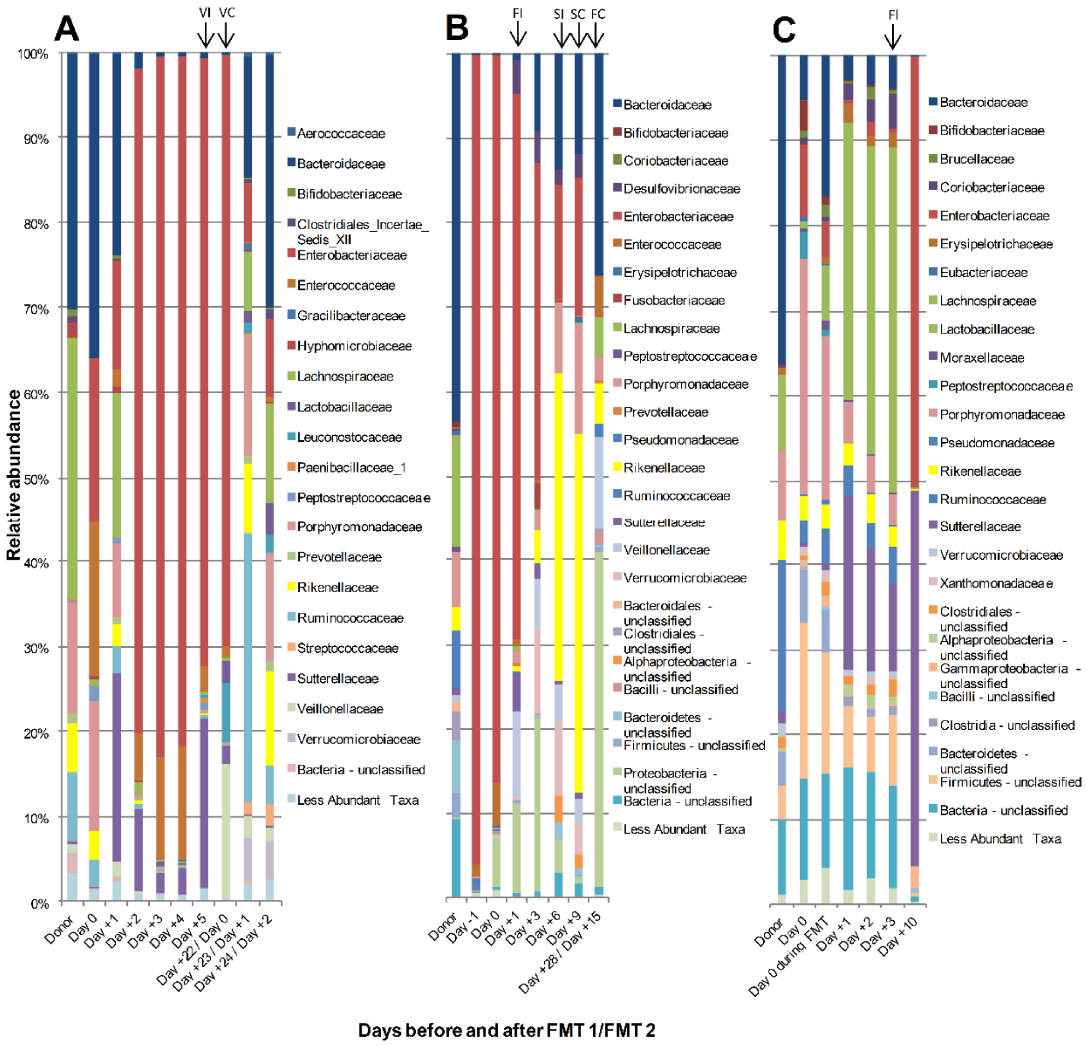


Figure 7.4 – Fecal bacterial families undergo change following each FMT. Relative abundances of bacterial families in fecal samples across treatment course in Patient 2 (A), Patient 3 (B), and Patient 4 (C). Colors correspond to families (see key in figure). Legend: Day 0 during FMT = sample taken from colonoscopy flush prior to FMT; less abundant taxa = less than 0.5% of total sequences. Numbers before slash indicate days after FMT 1; numbers after slash indicate days after FMT 2. Arrows indicate initiation or cessation of antibiotics: VI = vancomycin initiation, VC = vancomycin cessation, FI = fidaxomicin initiation, FC = fidaxomicin cessation, SI = trimethoprim-sulfamethoxazole (TMP-SMX) initiation, SC = TMP-SMX cessation (see Materials and Methods).

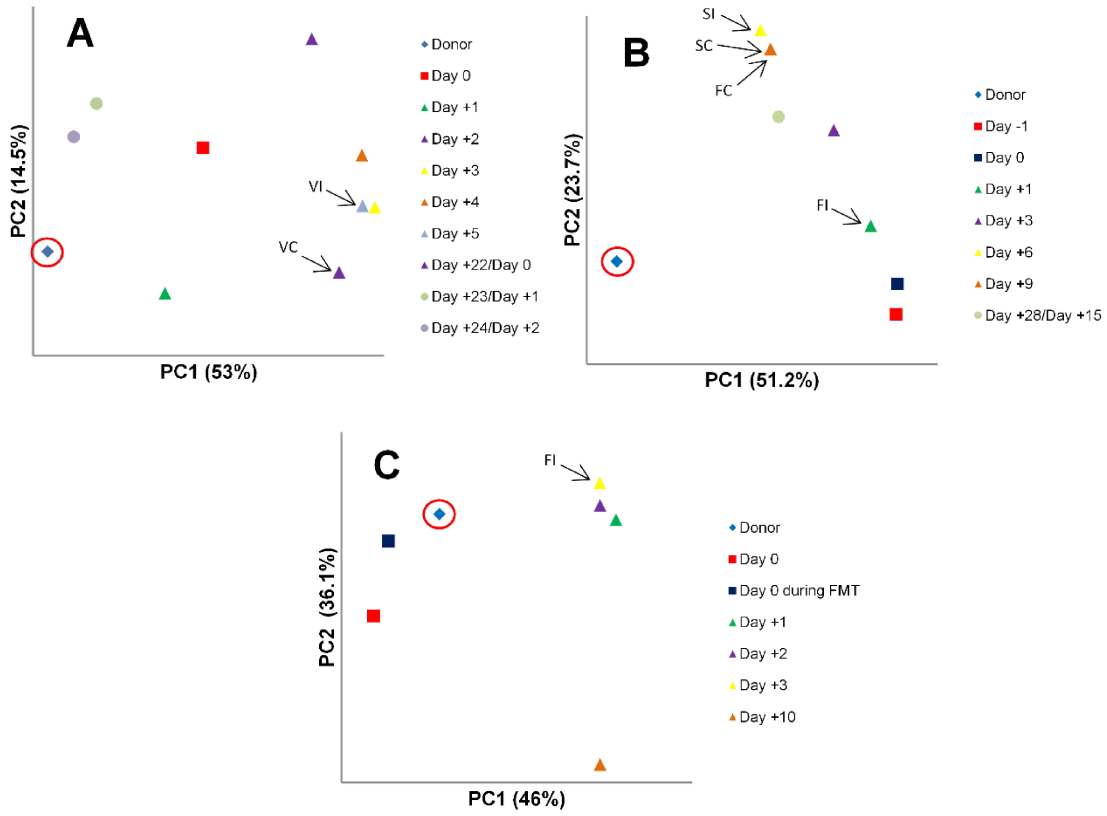


Figure 7.5 - Fecal bacterial communities shift relative to donor communities.

Principal coordinate analysis plots generated from weighted, normalized UniFrac distances for Patient 2 (A), Patient 3 (B), and Patient 4 (C). Legend: Day 0 during FMT = sample taken from colonoscopy flush prior to FMT; PC = principal coordinate; percent variation explained follows coordinate; and red circles indicate donor samples. Numbers before slash indicate days after FMT 1; numbers after slash indicate days after FMT 2. Arrows indicate initiation or cessation of antibiotics: VI = vancomycin initiation, VC = vancomycin cessation, FI = fidaxomicin initiation, FC = fidaxomicin cessation, SI = trimethoprim-sulfamethoxazole (TMP-SMX) initiation, SC = TMP-SMX cessation (see Materials and Methods).

Chapter 8

Discussion

Clostridium difficile infection and fecal microbiota transplantation

Clostridium difficile infection (CDI) is an increasingly common and severe nosocomial infection (Zilberberg et al., 2008; Miller et al., 2011; Markelov et al., 2011; Dubberke and Olsen, 2012; Magill et al., 2014; Lessa et al., 2015). With the rise in CDI, the incidence of recurrences of this infection, where symptoms and the presence of the pathogen in the stool return after completion of antibiotic therapy, is also on the rise (Pepin et al., 2005; Kelly and Lamont, 2008; Khoruts and Sadowsky, 2011; Borody and Khoruts, 2011; Vardakas et al., 2012; Lessa et al., 2015). Unfortunately, each recurrence drastically increases the chance of further recurrence; initial recurrences occur in 20-30% of patients treated with recommended antibiotics (Khoruts and Sadowsky, 2011; Vardakas et al., 2012), while additional recurrences can occur in up to 65% of patients who have already had one recurrent infection (Khoruts and Sadowsky, 2011). Eventually, these patients may enter a vicious cycle characterized by repeated antibiotic therapy followed by recurrence of CDI once antibiotics are halted – a condition known as recurrent CDI syndrome (R-CDI).

Although most R-CDI patients are refractory towards antibiotic therapy, treatment via transplantation with fecal material from a healthy donor is increasingly used to treat these individuals. Compared to standard antibiotic therapy, fecal microbiota transplantation (FMT) is extremely effective, relieving the symptoms of R-CDI and preventing further recurrence in more than 90% of patients (Hamilton et al., 2012; Kassam et al., 2013). Despite the extraordinary efficacy of FMT, however, little is known about its mechanisms, limiting our ability to rationally design novel therapies for patients

who do not meet the criteria for or fail FMT, and limiting our understanding of the role our intestinal microbes play in our health.

Summary of findings

In this work we have outlined our efforts to identify potential mechanisms behind FMT, test those mechanisms, and use our understanding of those mechanisms to develop novel treatments for R-CDI. Several previous efforts have used 16S rRNA gene sequencing and subsequent bacterial community profiling to understand changes in the fecal microbiome after the procedure. The results of these efforts have indicated that the fecal microbiome undergoes significant shifts after FMT: while fecal communities from patients with R-CDI are dominated by *Proteobacteria*, following FMT these communities are predominantly *Bacteroidetes* and *Firmicutes* (Khoruts et al., 2010; Shahinas et al., 2012; Hamilton et al., 2013; van Nood et al., 2013; Song et al., 2013; Weingarden et al., 2014; Seekatz et al., 2014; Fuentes et al., 2014), similar to the communities found in donors as well as in healthy individuals (Zoetendal et al., 2008; Arumugam et al., 2011). However, these studies give very little functional data on the fecal microbiome, and furthermore focus on the shift from pre-FMT to post-FMT communities, with little information on the stability and normalization of the fecal microbiome in the long- and short-term following the procedure.

One previous study has closely examined the dynamics of the fecal microbial community following FMT (Song et al., 2013). These authors concluded that because changes in the microbiome were observable after FMT, the community was unstable for at least six weeks following the procedure (Song et al., 2013). However, it is well-known that the fecal microbiome can undergo changes in healthy individuals over time (Dethlefsen et al., 2008; Dethlefsen and Relman, 2011), and therefore simply observing

changes post-FMT is not an indication that the microbiome has not stabilized. In our efforts to understand the mechanisms of FMT, we began by examining whether these changes in the microbiota fell within parameters comparable to changes in healthy individuals (Chapter 2).

In order to understand the changes in the microbiome following FMT, we compared microbial community shifts in patients to shifts within donor samples, and additionally compared patient's fecal communities to those of healthy individuals as described in the Human Microbiome Project (Turnbaugh et al., 2007) (Chapter 2). Our findings revealed that while the composition of the fecal microbiota continued to change after FMT, the fecal communities of patients remained similar to those found in healthy individuals. Furthermore, although the patients' microbiota continued to shift after FMT, the fecal microbiota of the donor underwent comparable shifts over time. Finally, we defined two new parameters, normalization and dynamic range, to describe these post-FMT changes. Neither normalization nor dynamic range were significantly different in patients compared to the donor. Overall, our findings suggest that although fecal bacterial communities of patients after FMT continue to change, these changes fall within the normal variation of healthy individuals.

While these findings provided key insight into the engraftment of fecal bacteria and may be useful as a biomarker to judge recovery versus recurrence after FMT, they still do not provide functional data explaining how the engrafted microbiome prevents *C. difficile* recurrence. To understand potential functions of the transplanted microbiota, we performed metabolomics analysis of patient fecal samples before and after transplant, along with analysis of the fecal bacterial communities (Chapter 3). Our findings revealed

that, concurrent with recovery of the fecal microbiota, fecal bile acid composition normalized. Specifically, bile acids present in patients' fecal samples prior to FMT were dominated by primary bile acids, produced by the liver, while bile acids present after FMT as well as in healthy donors were predominantly secondary bile acids, which are produced from primary bile acids by native colonic bacteria (Wells et al., 2000; Wells et al., 2003; Ridlon et al., 2006; Hoffman and Hagey, 2008).

Importantly, previous work has identified a possible link between bile acids and the physiology of *Clostridium difficile*. Some primary bile acids, notably cholate and its conjugate taurocholate, have been shown to cause *C. difficile* spores to germinate (Wilson, 1983; Sorg and Sonenshein, 2008). In contrast, several secondary bile acids, including lithocholic acid, inhibit germination (Sorg and Sonenshein, 2009; Sorg and Sonenshein, 2010). We hypothesized, therefore, that the shift in fecal bile acid composition we observed after FMT could represent a change from an environment favorable towards germination towards an environment where germination could not occur, and therefore was a mechanism explaining the success of FMT.

Despite this enticing hypothesis, the relationship between primary and secondary bile acids and *C. difficile* is not completely cut-and-dried. In particular, the primary bile acid chenodeoxycholic acid (CDCA) can inhibit spore germination (Sorg and Sonenshein, 2009; Sorg and Sonenshein, 2010), while the secondary bile acid deoxycholate (DCA) has been shown to induce germination (Wilson, 1983; Sorg and Sonenshein, 2008). To complicate the situation further, the response of different strains to CDCA can vary: while spores from most strains are inhibited from germinating with CDCA, it has no effect on germination in some strains, and in a few strains CDCA can

itself induce germination (Heeg et al., 2012). Therefore, it was important to test our hypothesis using combinations of bile acids reflective of the fecal environment before and after FMT, rather than simply individual bile acids, and it was also necessary to investigate the response of a variety of strains of *C. difficile* to these bile acid combinations.

Our results with bile acid combinations revealed that exposure to fecal bile acids present before FMT induced germination of *C. difficile* spores, while exposure to bile acids present after FMT did not (Chapter 4), supporting our hypothesis. Furthermore, we demonstrated that the post-FMT bile acid combination significantly inhibited vegetative growth of *C. difficile* compared to pre-FMT bile acids. Importantly, our results remained similar across a variety of *C. difficile* isolates and strains. These findings suggest that while individual bile acids may have opposing effects on *C. difficile* physiology, the overall fecal bile acid composition of patients with R-CDI allows for *C. difficile* germination and growth, while fecal bile acid composition after FMT prevents both germination and growth. This shift in fecal bile acid composition following FMT, therefore, likely prevents CDI recurrence and explains the success of the procedure.

Having provided strong evidence for a mechanism underlying FMT, we sought to use our understanding of this mechanism to rationally design novel therapies for R-CDI. One secondary bile acid, ursodeoxycholic acid (UDCA), is already approved for clinical use for bile acid disorders (Poupon, 2010; Lindor et al., 2009) and has been shown in a limited study to inhibit *in vitro C. difficile* germination (Sorg and Sonenshein, 2010). Here we demonstrated that UDCA could inhibit *in vitro* germination, as well as growth,

across the multiple strains we examined previously, suggesting that this drug could be used as a new therapy to prevent recurrence of CDI.

Despite the potential of UDCA, however, the drug has one key flaw: it is absorbed in the small intestine and enters the enterohepatic circulation, limiting its intracolonic concentration (Rodrigues et al., 1995; Hofmann and Hagey, 2008). However, certain derivatives of UDCA, notably C7-sulfated UDCA (7-SUDCA), have been shown to be minimally absorbed in the small intestine in animal models (Rodrigues et al., 1995). We therefore tested whether 7-SUDCA, like UDCA, could prevent *C. difficile* germination. Our results demonstrated that at concentrations ranging from 2-5 mM, 7-SUDCA significantly inhibited germination of all tested isolates of *C. difficile*. As these concentrations are well below the intracolonic concentrations of the drug in a rat model (Rodrigues et al., 1995), 7-SUDCA is therefore a promising novel therapeutic to prevent the recurrence of CDI.

Because our *in vitro* results suggested that 7-SUDCA could be a novel therapy for CDI, we next tested the efficacy of this drug *in vivo* using a mouse model. We successfully generated a model of CDI which displayed significant weight loss following infection and maintained productive infection based on *C. difficile* growth from the feces up to five days following infection. Using this model, we demonstrated that 7-SUDCA treatment prevented weight loss and delayed the appearance of the bacterium in the feces. Although 7-SUDCA was not delivered as initially intended, these efforts indicate that the drug is likely to be a successful therapy for preventing CDI. Future efforts using this model will be focused on twice-daily delivery of the drug to ensure more stable concentrations of 7-SUDCA in the colon, delivery immediately following infection to

mimic previous work with similar drugs (Howerton et al., 2013), and the addition of a drug-treated group which is not infected to insure that the abrogation of weight loss with the drug is not related to weight gain by the drug. While our findings with 7-SUDCA *in vivo* are preliminary, they are promising results which suggest that the drug is effective and that translation into humans is a very real possibility.

Although the use of UDCA itself may be limited due to its efficient uptake into enterohepatic circulation in the small intestine (Rodrigues et al., 1995; Hofmann and Hagey, 2008), in patients with altered intestinal anatomy the drug may have utility. *C. difficile* infection is associated with 10% of diarrheal symptoms in patients with an ileal pouch reservoir that is surgically created after a total colectomy (Seril and Shen, 2014). Unfortunately, FMT may not be a reliable treatment for these patients (Hamilton et al., 2012; Borody et al., 2014; Patel et al., 2014), possibly because the typical microbiota of the small intestine and ileal pouch is distinct from that of the colon (Booijink et al., 2010; Zella et al., 2011; Zoetendal et al., 2012; Young et al., 2013; Yasuda et al., 2015). We successfully treated one such patient with oral UDCA, finding that in addition to remaining free of infection more than eight months after halting antibiotics, the concentrations of UDCA in the patient's feces were more than sufficient to inhibit germination of spores generated from the patient's own isolate of *C. difficile* (Chapter 6).

Despite our clinical success, however, the fecal microbiota of this patient did not normalize over three months after beginning UDCA. In particular, the *Bacteroidetes* phylum, normally a dominant member of the fecal microbiota (Zoetendal et al., 2008; Arumugam et al., 2011), remained nearly absent, the α-diversity of the microbiota remained low, and the overall fecal bacterial communities remained distinct from healthy

individuals. Unlike patients who successfully recover following FMT (Khoruts et al., 2010; Shahinas et al., 2012; Hamilton et al., 2013; van Nood et al., 2013; Song et al., 2013; Weingarden et al., 2014; Seekatz et al., 2014; Fuentes et al., 2014), the microbiota seems not to have recovered in this patient. Therefore, it may be necessary to continue oral UDCA treatment to prevent recurrence of CDI.

In addition to exploring novel therapies for R-CDI, we also presented here work on using FMT for other forms of CDI (Chapter 7). As CDI has increased in incidence, the incidence of severe or complicated forms of CDI has also risen (McDonald et al., 2005; Kelly and Lamont, 2008; Merrigan et al., 2010). These forms of CDI are typically clinically defined, using criteria such as the ATLAS criteria which include age, temperature, leukocytosis, low serum albumin, and concurrent antibiotic use for other conditions (Mulherin et al., 2014), though they can also include serious sequelae such as toxic megacolon or pseudomembranous colitis (Cohen et al., 2010). Unfortunately, currently recommended therapy for these severe forms of CDI is colectomy, or removal of the colon (Markelov et al., 2011), which is associated with a postoperative mortality rate of 35-80% (Dallal et al., 2002; Koss et al., 2006; Wysowski, 2006; Lamontagne et al., 2007). We examined whether FMT could be used as an alternative therapy to colectomy for four cases of severe CDI, and found that two sequential FMTs could lead to lasting cure for the patients. An initial FMT clinically stabilizes the patient, while a second FMT performed two weeks after the first can insure stable engraftment of donor microbiota and prevent recurrence of disease.

Although these results suggest that FMT is a useful alternative to colectomy in severe CDI, analysis of the fecal microbiome of these patients indicates that the

mechanisms of the procedure may be different than those which prevent R-CDI. While a single FMT did not result in sustained resistance to CDI recurrence, as indicated by both the maintained predominance of the *Enterobacteriaceae* and recurrence of disease following the procedure, clinical recovery of the patients, including defervesence, decreased white blood cell count, and hemodynamic stabilization, was noted after the initial procedure. In addition to non-lasting changes to the *Enterobacteriaceae*, the *Lachnospiraceae*, often associated with recovery in R-CDI patients following FMT (Hamilton et al., 2013; van Nood et al., 2013; Song et al., 2013; Weingarden et al., 2014; Seekatz et al., 2014; Fuentes et al., 2014), recovered only temporarily (for 1 day or less) following initial FMT. Previous results have indicated that the *Lachnospiraceae* are associated with the mucosal surface of healthy individuals and may positively influence gut health, via regulation of the immune system and production of molecules which act as fuel for colonic epithelial cells (Pryde et al., 2002; Hamer et al., 2008; Atarashi et al., 2011; Nava and Stappenbeck, 2011). It is possible, therefore, that this brief reappearance of the *Lachnospiraceae* may have decreased the inflammatory response in these patients, allowing for clinical recovery, even if lasting resistance to CDI recurrence was not achieved.

Concluding remarks and future directions

Although FMT is an extremely successful treatment for R-CDI, its mechanisms were heretofore unknown. This work represents our efforts to uncover the mechanisms behind this treatment and subsequently design novel therapies based on these mechanisms. These therapies may be used to treat patients who fail FMT, who do not meet the criteria for FMT, or who choose not to have an FMT. Ultimately, these new therapies may replace FMT entirely.

Our findings reveal that fecal bile acids play a key role in preventing subsequent recurrence following FMT (Fig. 8.1). Before FMT, bile acids in the feces are predominantly primary bile acids, which in combination induce *C. difficile* germination and permit vegetative growth. In contrast, after FMT fecal bile acids are dominated by the secondary bile acids, which in combination do not allow germination and significantly inhibit vegetative growth. Furthermore, pharmacological manipulation of these bile acids is capable of preventing *C. difficile* infection. For patients who develop R-CDI pouchitis following colectomy, UDCA is already available as a therapy to prevent recurrence. We have demonstrated *in vitro* and have preliminary data from a mouse model of CDI that 7-SUDCA can prevent germination and infection. 7-SUDCA represents one bile acid derivative that may be useful in patients with normal intestinal anatomy.

In addition to 7-SUDCA, it is likely that other bile acid analogs may represent additional therapeutic options. Much of our future work will focus on testing other bile acid analogs, some of which may have even greater effects on *C. difficile*. Although 7-

SUDCA has already been shown to be unabsorbed by the host and non-metabolized by the colonic microbiota, pharmacokinetic studies will be needed to determine whether these other analogs are also unabsorbed and non-metabolized.

While we have made significant strides towards non-antibiotic pharmacological solutions to R-CDI, manipulation of the microbiota remains a viable avenue of treatment. Our work and the work of others (Buffie et al., 2014) have pointed towards the potential of bacteria capable of performing 7α-dehydroxylation to produce secondary bile acids *in vivo*. While Buffie and colleagues focused on *Clostridium scindens*, one of the best-described 7α-dehydroxylating bacteria, it is known that other bacteria in the human colon possess this metabolic pathway. Future efforts will be focused on uncovering and understanding these bacteria, and investigating their use in preventing CDI recurrence.

Finally, having demonstrated the efficacy of 7-SUDCA *in vitro* and in mice, it will be necessary to translate this novel therapy to the clinic. This will require multidisciplinary efforts from clinical researchers, microbiologists, medicinal chemists, and more. Eventually, it is possible that 7-SUDCA or another bile acid analog may replace FMT entirely, as the ease of oral therapy makes pharmacological solutions preferable to the procedure. The work presented herein represents the first steps down this path.

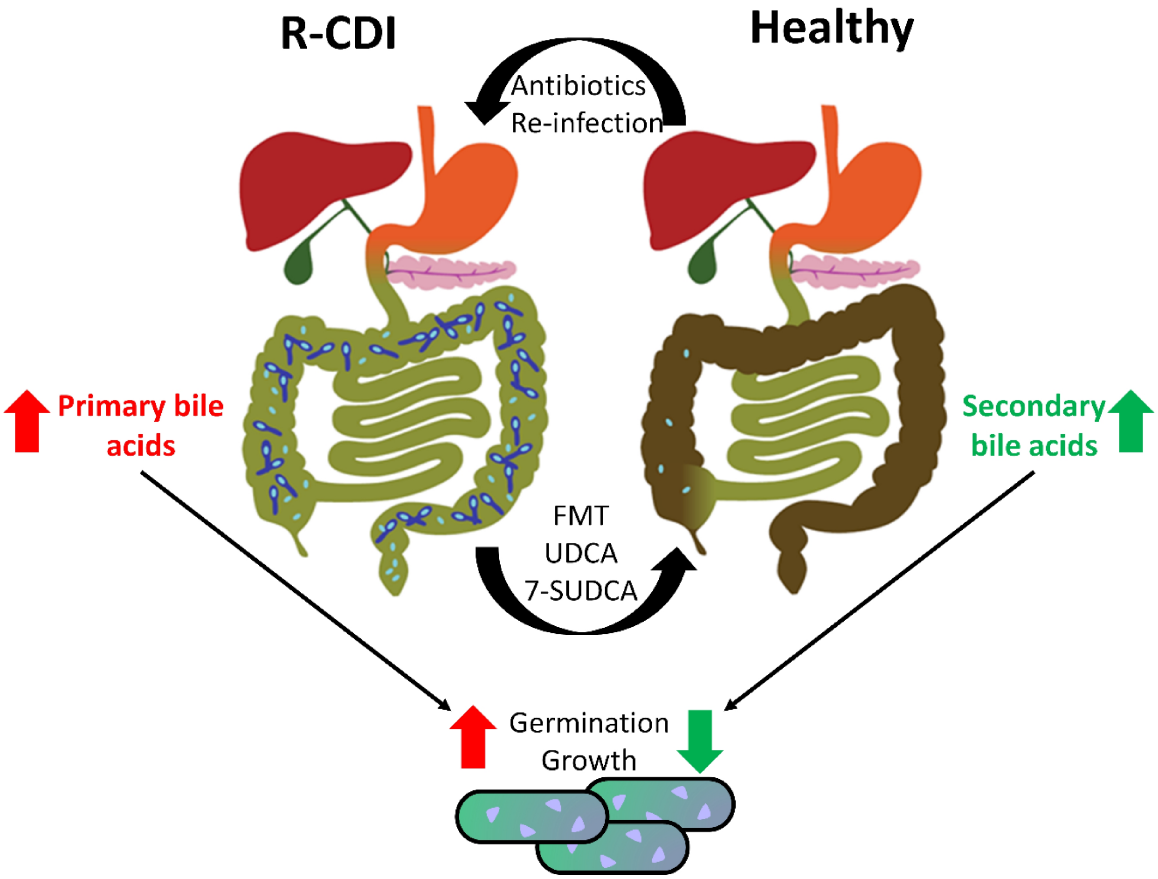


Figure 8.1 – Model of the mechanism of fecal microbiota transplantation and the relationship between bile acids and *C. difficile* infection. R-CDI is characterized by an abundance of primary bile acids in the feces, which induce germination and allow growth of *C. difficile*. In contrast, the feces of healthy individuals, or post-FMT patients, is dominated by secondary bile acids, which do not induce germination and limit growth of *C. difficile*. Predominance by primary bile acids in the feces may be caused or maintained by the repeated antibiotic treatments R-CDI patients receive, while FMT restores secondary bile acid metabolism to prevent recurrence of infection. Prevention of recurrence may also be achieved by directly replacing secondary bile acids using UDCA or 7-SUDCA. R-CDI: recurrent *C. difficile* infection; FMT: fecal microbiota transplantation; UDCA: ursodeoxycholic acid; 7-SUDCA: C7-sulfated UDCA.

Bibliography

Aas J, Gessert CE, Bakken JS. 2003. Recurrent *Clostridium difficile* colitis: case series involving 18 patients treated with donor stool administered via a nasogastric tube. *Clin Infect Dis* **36**:580-585.

Alberti KG, Zimmet P, Shaw J. 2005. The metabolic syndrome – a new worldwide definition. *Lancet* **366**:1059-1062.

Ananthakrishnan AN. 2011. *Clostridium difficile* infection: epidemiology, risk factors and management. *Nat Rev Gastroenterol Hepatol* **8**:17-26.

Arroyo LG, Rousseau J, Willey BM, Low DE, Staempfli H, McGeer A, Weese JS. 2005. Use of a selective enrichment broth to recover *Clostridium difficile* from stool swabs stored under different conditions. *J Clin Microbiol* **43**:5341-5343.

Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, Fernandes GR, et al. 2011. Enterotypes of the human gut microbiome. *Nature* **473**:174-180.

Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y, Cheng G, et al. 2011. Induction of colonic regulatory T cells by indigenous *Clostridium* species. *Science* **331**:337-341.

Bäckhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI. 2005. Host-bacterial mutualism in the human intestine. *Science* **307**:1915-1920.

Bakken JS, Borody T, Brandt LJ, Brill JV, Demarco DC, Franzos MA, Kelly C, et al. 2011. Treating *Clostridium difficile* infection with fecal microbiota transplantation. *Clin Gastroenterol Hepatol* **9**:1044-1049.

Bliss DZ, Johnson S, Clabots CR, Savik K, Gerding DN. 1997. Comparison of cycloserine-cefoxitin-fructose agar (CCFA) and taurochoalte-CCFA for recovery of *Clostridium difficile* during surveillance of hospitalized patients. *Diagn Microbiol Infect Dis* **29**:1-4.

Bokulich NA, Subramanian S, Faith JJ, Gevers D, Gordon JI, Knight R, Mills DA, Caporaso JG. 2013. Quality-filtering vastly improves diversity estimates form Illumina amplicon sequencing. *Nature Methods* **10**:57-59.

Booijink CC, El-Aidy S, Rajilić-Stojanović M, Heilig HG, Troost FJ, Smidt H, Kleerebezem M, et al. 2010. High temporal and inter-individual variation detected in the human ileal microbiota. *Environ Microbiol* **12**:3213-3227.

Borody TJ, Warren EF, Leis S, Surace R, Ashman O. 2003. Treatment of ulcerative colitis using fecal bacteriotherapy. *J Clin Gastroenterol* **37**:42-47.

Borody TJ, Khoruts A. 2011. Fecal microbiota transplantation and emerging applications. *Nat Rev Gastroenterol Hepatol* **9**:88-96.

Borody TJ, Leis S, Pang G, Wettstein AR. 2014. Fecal microbiota transplantation in the treatment of recurrent *Clostridium difficile* infection. UpToDate, accessed January 15, 2015.

Brandt LJ, Borody TJ, Campbell J. 2011. Endoscopic fecal microbiota transplantation: "first-line" treatment for severe *Clostridium difficile* infection? *J Clin Gastroenterol* **45**:655-657.

Braun V, Hundsberger T, Leukel P, Sauerborn M, von Eichel-Streiber C. 1996. Definition of the single integration site of the pathogenicity locus in *Clostridium difficile*. *Gene* **181**:29-38.

Britton RA, Young VB. 2012. Interaction between the intestinal microbiota and host in *Clostridium difficile* colonization resistance. *Trends Microbiol* **20**:313-319.

Brown CT, Davis-Richardson AG, Giongo A, Gano KA, Crabb DB, Mukherjee N, Casella G, et al. 2011. Gut microbiome metagenomics analysis suggests a functional model for the development of autoimmunity for type 1 diabetes. *PLoS ONE* **6**:e25792.

Buggy BP, Hawkins CC, Fekety R. 1985. Effect of adding sodium taurocholate to selective media on the recovery of *Clostridium difficile* from environmental surfaces. *J Clin Microbiol* **21**:636-637.

Buffie CG, Bucci V, Stein RR, McKenney PT, Ling L, Gobourne A, No D, et al. 2014. Precision microbiome reconstitution restores bile acid mediated resistance to *Clostridium difficile*. *Nature* **517**:205-208.

Bylesjo M, Rantalainen M, Nicholson JK, Holmes E, Trygg J. 2008. K-OPLS package: kernel-based orthogonal projections to latent structures for prediction and interpretation in feature space. *BMC Bioinformatics* **9**:106.

Carroll KC, Bartlett JG. 2011. Biology of *Clostridium difficile*: implications for epidemiology and diagnosis. *Annu Rev Microbiol* **65**:501-521.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, et al. 2010. QIIME allows analysis of high-throughput community sequencing data. *Nature Methods* **7**:335-336.

Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, Owens SM, et al. 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J* **6**:1621-1624.

Carman RJ, Simon MA, Petzold HE, Wimmer RF, Batra MR, Fernandez AH, Miller MA, Bartholomew M. 2005. Antibiotics in the human food chain: establishing no effect levels of tetracycline, neomycin, and erythromycin using a chemostat model of the human colonic microflora. *Regul Toxicol Pharmacol* **43**:168-180.

Chang JY, Antonopoulos DA, Kalra A, Tonelli A, Khalife WT, Schmidt TM, Young VB. 2008. Decrease diversity of the fecal microbiome in recurrent *Clostridium difficile*-associated diarrhea. *J Infect Dis* **197**:435-438.

Chen C, Ma X, Malfatti MA, Krausz KW, Kimura S, Felton JS, Idle JR, Gonzalez FJ. 2007. A comprehensive investigation of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) metabolism in the mouse using a multivariate data analysis approach. *Chem Res Toxicol* **20**:531-542.

Chitnis AS, Holzbauer SM, Belflower RM, Winston LG, Bamberg WM, Lyons C, Farely MM, et al. 2013. Epidemiology of community-associated *Clostridium difficile* infection, 2009 through 2011. *JAMA Intern Med* **173**:1359-1367.

Cohen SH, Gerding DN, Johnson S, Kelly CP, Loo VG, McDonald LC, Pepin J, Wilcox MH. 2010. Clinical practice guidelines for *Clostridium difficile* infection in adults: 2010 update by the Society for Healthcare Epidemiology of America (SHEA) and the Infectious Diseases Society of America (IDSA). *Infect Control Hosp Epidemiol* **31**:431-455.

Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, Kulam-Syed-Mohideen AS, et al. 2009. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Res* **37**:D141-D145.

Dallal RM, Harbrecht BG, Boujokas AJ, Sirio CA, Farkas LM, Lee KK, Simmons RL. 2002. Fulminant *Clostridium difficile*: an underappreciated and increasing cause of death and complications. *Ann Surg* **235**:363-372.

David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, Ling AV, et al. 2014. Diet rapidly and reproducibly alters the human gut microbiome. *Nature* **505**:559-563.

Deakin LJ, Clare S, Fagan RP, Dawson LF, Pickard DJ, West MR, Wren BW, et al. 2012. The *Clostridium difficile* spo0A gene is a persistence and transmission factor. *Infect Immun* **80**:2704-2711.

Dethlefsen L, Huse S, Sogin ML, Relman DA. 2008. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. *PLoS Biol* **6**:e280.

Dethlefsen L, Relman DA. 2011. Incomplete recovery and individualized responses of the human gut distal gut microbiota to repeated antibiotic perturbation. *Proc Natl Acad Sci USA* **108 Suppl 1**:4554-4561.

Doerner KC, Takamine F, LaVoie CP, Mallonee DH, Hylemon PB. 1997. Assessment of fecal bacteria with bile acid 7α-dehydroxylating activity for the presence of bai-like genes. *Appl Environ Microbiol* **63**:1185-1188.

Dubberke ER, Olsen MA. 2012. Burden of *Clostridium difficile* on the healthcare system. *Clin Infect Dis* **55**:S88-S92.

Duca FA, Sakar Y, Lepage P, Devime F, Langelier B, Doré J, Covasa M. 2014. Replication of obesity and associated signaling pathways through transfer of microbiota from obese-prone rats. *Diabetes* **63**:1624-1636.

Edgar RC. 2010. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26**:2460-2461.

Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. 2011. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* **27**:2194-2200.

Eiseman B, Silen W, Bascom GS, Kauvar AJ. 1958. Fecal enema as an adjunct in the treatment of pseudomembranous enterocolitis. *Surgery* **44**:854-859.

Faith JJ, Guruge JL, Charbonneau M, Subramanian S, Seedorf H, Goodman AL, Clemente JC, et al. 2013. The long-term stability of the human gut microbiota. *Science* **341**:1237439.

Fashner J, Garcia M, Ribble L, Crowell K. 2011. Clinical inquiry: what risk factors contribute to *C. difficile* diarrhea? *J Fam Pract* **60**:545-457.

Ferreyra JA, Wu KJ, Hryckowian AJ, Bouley DM, Weimer BC, Sonnenburg JL. 2014. Gut microbiota-produced succinate promotes *C. difficile* infection after antibiotic treatment or motility disturbance. *Cell Host Microbe* **16**:770-777.

Fimlaid KA, Bond JP, Schutz KC, Putnam EE, Leung JM, Lawley TD, Shen A. 2013. Global analysis of the sporulation pathway of *Clostridium difficile*. *PLoS Genet* **9**:e1003660.

Francis MB, Allen CA, Shrestha R, Sorg JA. 2013. Bile acid recognition by the *Clostridium difficile* germinant receptor, CspC, is important for establishing infection. *PLoS Pathog* **9**:e1003356.

Francis MB, Allen CA, Sorg JA. 2015. Spore cortex hydrolysis precedes DPA release during *Clostridium difficile* spore germination. *J Bacteriol* 2015 Apr 27. Pii: JB.02575-14 (Epub ahead of print).

Frank DN, St. Amand AL, Feldman RA, Boedeker EC, Harpaz N, Pace NR. 2007. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc Natl Acad Sci USA* **104**:13780-13785.

Fuentes S, van Nood E, Tims S, Heikamp-de Jong I, ter Braak CJ, Keller JJ, Zoetendal EG, de Vos WM. 2014. Reset of a critically disturbed microbial ecosystem: faecal transplant in recurrent *Clostridium difficile* infection. *ISME J* **8**:1621-1633.

Gallegos-Orozco JF, Paskvan-Gawryletz CD, Gurudu SR, Orenstein R. 2012. Successful colonoscopic fecal transplant for severe acute *Clostridium difficile* pseudomembranous colitis. *Rev Gastroenterol Mex* **77**:40-42.

Gasteiger E, Gattiker A, Hoogland C, Ivanyi I, Appel RD, Bairoch A. 2003. ExPASy: the proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Res* **31**:3784-3788.

Gevers D, Kugathasan S, Denson LA, Vazquez-Baeza Y, Van Treuren W, Ren B, Schwager E, et al. 2014. The treatment-naïve microbiome in new-onset Crohn's disease. *Cell Host Microbe* **15**:382-392.

Giel JL, Sorg JA, Sonenshein AL, Zhu J. 2010. Metabolism of bile salts in mice influences spore germination in *Clostridium difficile*. *PLoS ONE* **5**:e8740.

Giongo A, Gano KA, Crabb DB, Mukherjee N, Novelo LL, Casella G, Drew JC, et al. 2011. Toward defining the autoimmune microbiome for type 1 diabetes. *ISME J* **5**:82-91.

de Goffau MC, Luopajärvi K, Knip M, Ilonen J, Ruohtula T, Härkönen T, Orivuori L, et al. 2013. Fecal microbiota composition differs between children with β-cell autoimmunity and those without. *Diabetes* **62**:1238-1244.

Gough E, Shaikh H, Manges AR. 2011. Systematic review of intestinal microbiota transplantation (fecal bacteriotherapy) for recurrent *Clostridium difficile* infection. *Am J Gastroenterol* **107**:761-767.

Hall AJ, Curns AT, McDonald LC, Parashar UD, Lopman BA. 2012. The roles of *Clostridium difficile* and norovirus among gastroenteritis-associated deaths in the United States, 1999-2007. *Clin Infect Dis* **55**:216-223.

Hamer HM, Jonkers D, Venema K, Vanhoutvin S, Troost FJ, Brummer RJ. 2011. Review article: the role of butyrate on colonic function. *Aliment Pharmacol Ther* **27**:104-119.

Hamady M, Lozupone C, Knight R. 2010. Fast UniFrac: facilitating high-throughput phylogenetic analyses of microbial communities including analysis of pyrosequencing and PhyloChip data. *ISME J* **4**:17-27.

Hamilton MJ, Weingarden AR, Sadowsky MJ, Khoruts A. 2012. Standardized frozen preparation for transplantation of fecal microbiota for recurrent *Clostridium difficile* infection. *Am J Gastroenterol* **47**:735-737.

Hamilton MJ, Weingarden AR, Unno T, Khoruts A, Sadowsky MJ. 2013. High-throughput DNA sequence analysis reveals stable engraftment of gut microbiota following transplantation of previously frozen fecal bacteria. *Gut Microbes* **4**:125-135.

Hashimoto S, Igimi H, Uchida K, Satoh T, Benno Y, Takeuchi N. 1996. Effects of β-lactam antibiotics on intestinal microflora and bile acid metabolism in rats. *Lipids* **31**:601-609.

He M, Miyajima F, Roberts P, Ellison L, Pickard DJ, Martin MJ, Connor TR, et al. 2013. Emergence and global spread of epidemic healthcare-associated *Clostridium difficile*. *Nat Genet* **45**:109-113.

Heeg D, Burns DA, Cartman ST, Minton NP. 2012. Spores of *Clostridium difficile* clinical isolates display a diverse germination response to bile salts. *PLoS ONE* **7**:e32381.

Higgins D, Dworkin J. 2012. Recent progress in *Bacillus subtilis* sporulation. *FEMS Microbiol Rev* **36**:131-148.

Hirano S, Nakama R, Tamaki M, Masuda N, Oda H. 1981. Isolation and characterization of thirteen intestinal microorganisms capable of 7α-dehydroxylating bile acids. *Appl Environ Microbiol* **41**:737-745.

Hirsch BE, Saraiya N, Poeth K, Schwartz RM, Epstein ME, Honig G. 2015. Effectiveness of fecal-derived microbiota transfer using orally administered capsules for recurrent *Clostridium difficile* infection. *BMC Infect Dis* **15**:191.

Hofmann AF. 1999. The continuing importance of bile acids in liver and intestinal disease. *Arch Intern Med* **159**:2647.

Hofmann AF, Hagey LR. 2008. Bile acids: chemistry, pathochemistry, biology, pathobiology, and therapeutics. *Cell Mol Life Sci* **65**:2461-2483.

de Hoon MJ, Eichenberger P, Vitkup D. 2010. Hierarchical evolution of the bacterial sporulation network. *Curr Biol* **20**:R735-745.

Hopkins MJ, Macfarlane GT. 2003. Nondigestible oligosaccharides enhance bacterial colonization resistance against *Clostridium difficile in vitro*. *Appl Environ Microbiol* **69**:1920-1927.

Howerton A, Patra M, Abel-Santos E. 2013. A new strategy for the prevention of *Clostridium difficile* infection. *J Infect Dis* **207**:1498-1504.

Howerton A, Patra M, Abel-Santos E. 2013. Fate of ingested *Clostridium difficile* spores in mice. *PLoS ONE* **8**:e72620.

Hu MY, Katchar K, Kyne L, Maroo S, Tummala S, Dreisbach V, Xu H, et al. 2009. Prospective derivation and validation of a clinical prediction rule for recurrent *Clostridium difficile* infection. *Gastroenterology* **136**:1206-1214.

Jones BV, Begley M, Hill C, Gahan CG, Marchesi JR. 2008. Functional and comparative metagenomics analysis of bile salt hydrolase activity in the human gut microbiome. *Proc Natl Acad Sci USA* **105**:13580-13585.

Joyce SA, MacSharry J, Casey PG, Kinsella M, Murphy EF, Shanahan F, Hill C, Gahan CGM. 2014. Regulation of host weight gain and lipid metabolism by bacterial bile acid modification in the gut. *Proc Natl Acad Sci USA* **111**:7421-7426.

Kachrimanidou M, Malisiovas N. 2011. *Clostridium difficile* infection: a comprehensive review. *Crit Rev Microbiol* **37**:178-187.

Karlsson FH, Få F, Nookaew I, Tremaroli V, Fagerberg B, Petranovic D, Bäckhed F, Nielsen J. 2012. Symptomatic atherosclerosis is associated with an altered gut metagenome. *Nat Commun* **3**:1245.

Karlsson FH, Tremaroli V, Nookaew I, Bergström G, Behre CJ, Fagerberg B, Nielsen J, Bäckhed F. 2013. Gut metagenome in European women with normal, impaired, and diabetic glucose control. *Nature* **498**:99-103

Kassam Z, Lee CH, Yuan Y, Hunt RH. 2013. Fecal microbiota transplantation for *Clostridium difficile* infection: systematic review and meta-analysis. *Am J Gastroenterol* **108**:500-508.

Kawamoto S, Maruya M, Kato LM, Suda W, Atarashi K, Doi Y, Tsutsui Y, et al. 2014. Foxp3$^+$ T cells regulate immunoglobulin A selection and facilitate diversification of bacterial species responsible for immune homeostasis. *Immunity* **41**:152-165.

Kelly CP, Lamont JT. 2008. *Clostridium difficile* – more difficult than ever. *N Engl J Med* **359**:1932-1940.

Kelly CR, de Leon L, Jasutkar N. 2012. Fecal microbiota transplantation for relapsing *Clostridium difficile* infection in 26 patients: methodology and results. *J Clin Gastroenterol* **46**:145-149.

Kelly CR, Ihunnah C, Fischer M, Khoruts A, Surawicz C, Afzali A, Aroniadis O, et al. 2014. Fecal microbiota transplant for treatment of *Clostridium difficile* infection in immunocompromised patients. *Am J Gastroenterol* **109**:1065-1071.

Khanna S, Pardi DS, Aronson SL, Kammer PP, Orenstein R, St. Sauver JL, Harmsen WS, Zinsmeister AR. 2012. The epidemiology of community-acquired *Clostridium difficile* infection: a population-based study. *Am J Gastroenterol* **107**:89-95.

Khoruts A, Dicksved J, Jansson JK, Sadowsky MJ. 2010. Changes in the composition of the human fecal microbiome after bacteriotherapy for recurrent *Clostridium difficile*-associated diarrhea. *J Clin Gastroenterol* **44**:354-360.

Khoruts A, Sadowsky MJ. 2011. Therapeutic transplantation of the distal gut microbiota. *Mucosal Immunol* **4**:4-7.

Khoruts A, Weingarden AR. 2014. Emergence of fecal microbiota transplantation as an approach to repair disrupted microbial gut ecology. *Immunol Lett* **162**:77-81.

Khoruts A, Sadowsky MJ, Hamilton MJ. 2015. Development of fecal microbiota transplantation suitable for mainstream medicine. *Clin Gastroenterol Hepatol* **13**:246-250.

Killgore G, Thompson A, Johnson S, Brazier J, Kuijper E, Pepin J, Frost EH, et al. 2008. Comparison of seven techniques for typing international epidemic strains of *Clostridium difficile*: restriction endonuclease analysis, pulsed-field gel electrophoresis, PCR-ribotyping, multilocus sequence typing, multilocus variable-number tandem-repeat analysis, amplified fragment length polymorphism, and surface layer protein A gene sequencing typing. *J Clin Microbiol* **46**:431-437.

Kim KH, Fekety R, Batts DH, Brown D, Cudmore M, Silva J, Waters D. 1981. Isolation of *Clostridium difficile* from the environment and contacts of patients with antibiotic-associated colitis. *J Infect Dis* **143**:42-50.

Kitahara M, Takamine F, Imamura T, Benno Y. 2000. Assignment of Eubacterium sp. VPI 12708 and related strains with high bile acid 7α-dehydroxylating activity to *Clostridium scindens* and proposal of *Clostridium hylemonae* sp. nov., isolated from human faeces. *Int J Syst Evol Microbiol* **50**:971-978.

Knights D, Silverberg MS, Weersma RK, Gevers D, Dijkstra G, Huang H, Tyler AD, et al. 2014. Complex host genetics influence the microbiome in inflammatory bowel disease. *Genome Med* **6**:107.

Koenigsknecht MJ, Theriot CM, Bergin IL, Schumacher CA, Schloss PD, Young VB. 2014. Dynamics and establishment of *Clostridium difficile* infection in the murine gastrointestinal tract. *Infect Immun* **83**:934-941.

Koren O, Goodrich JK, Cullender TC, Spor A, Laitinen K, Bäckhed HK, Gonzalez A, et al. 2012. Host remodeling of the gut microbiome and metabolic changes during pregnancy. *Cell* **150**:470-480.

Koss K, Clark MA, Sanders DS, Morton D, Keighley MR, Goh J. 2006. The outcome of surgery in fulminant *Clostridium difficile* colitis. *Colorectal Dis* **8**:149-154.

Kostic AD, Gevers D, Siljander H, Vatanen T, Hyötyläinen T, Hämäläinen A-M, Peet A, et al. 2015. The dynamics of the human infant gut microbiome in development and in progression toward type 1 diabetes. *Cell Host Microbe* **17**:260-273.

Lamontagne F, Labbe AC, Haeck O, Lesur O, Lalancette M, Patino C, Leblanc M, Laverdiere M, Pepin J. 2007. Impact of emergency colectomy on survival of patients with fulminant *Clostridium difficile* colitis during an epidemic caused by a hypervirulent strain. *Ann Surg* **245**:267-272.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, et al. 2007. Clustal W and Clustal X version 2.0. *Bioinformatics* **23**:2947-2948.

Lawley TD, Clare S, Walker AW, Goulding D, Stabler RA, Croucher N, Mastroeni P, et al. 2009. Antibiotic treatment of *Clostridium difficile* carrier mice triggers a supershedder state, spore-mediated transmission, and severe disease in immunocompromised hosts. *Infect Immun* **77**:3661-3669.

Lawley TD, Clare S, Deakin LJ, Goulding D, Yen JL, Raisen C, Brandt C, et al. 2010. Use of purified *Clostridium difficile* spores to facilitate evaluation of health care disinfection regimens. *Appl Environ Microbiol* **76**:6895-6900.

Lawley TD, Clare S, Walker AW, Stares MD, Connor TR, Raisen C, Goulding D, et al. 2012. Targeted restoration of the intestinal microbiota with a simple, defined bacteriotherapy resolves relapsing *Clostridium difficile* disease in mice. *PLoS Pathog* **8**:e1002995.

Lessa FC, Gould CV, McDonald LC. 2012. Current status of *Clostridium difficile* infection epidemiology. *Clin Infect Dis* **55 Suppl 2**:S65-S70.

Lessa FC, Mu Y, Winston LG, Dumyati GK, Farley MM, Beldavs ZG, Kast K, et al. 2014. Determinants of *Clostridium difficile* infection incidence across diverse United States geographic locations. *Open Forum Infect Dis* **1**:ofu048.

Lessa FC, Mu Y, Bamberg WM, Beldavs ZG, Dumyati GK, Dunn JR, Farley MM, et al. 2015. Burden of *Clostridium difficile* infection in the United States. *New Engl J Med* **372**:825-834.

Ley RE, Turnbaugh PJ, Klein S, Gordon JI. 2006. Human gut microbes associated with obesity. *Nature* **444**:1022-1023.

Lindor KD, Gershwin ME, Poupon R, Kaplan M, Bergasa NV, Heathcote EJ, American Association for Study of Liver. 2009. Primary biliary cirrhosis. *Hepatology* **50**:291-308.

Linevsky JK, Pothoulakis C, Keates S, Warny M, Keates AC, Lamont JT, Kelly CP. 1997. IL-8 release and neutrophil activation by *Clostridium difficile* toxin-exposed human monocytes. *Am J Physiol* **273**:G1333-G1340.

Loo VG, Poirier L, Miller MA, Oughton M, Libman MD, Michaud S, Bourgault AM, et al. 2005. A predominantly clonal multi-institutional outbreak of *Clostridium difficile*-associated diarrhea with high morbidity and mortality. *New Engl J Med* **353**:2442-2449.

Louie TJ, Miller MA, Mullane KM, Weiss K, Lentnek A, Golan Y, Gorbach S, et al. 2011. Fidaxomicin versus vancomycin for *Clostridium difficile* infection. *N Engl J Med* **364**:422-431.

Louie TJ, Cannon K, Byrne B, Emery J, Ward L, Eyben M, Krulicki W. 2012. Fidaxomicin preserves the intestinal microbiome during and after treatment of *Clostridium difficile* infection (CDI) and reduces both toxin reexpression and recurrence of CDI. *Clin Infect Dis* **55**:S132-S142.

Lozupone C, Knight R. 2005. UniFrac: a new phylogenetic method for comparing microbial communities. *Appl Environ Microbiol* **71**:8228-8235.

Lozupone CA, Li M, Campbell TB, Flores SC, Linderman D, Gebert MJ, Knight R, et al. 2013. Alterations in the gut microbiota ssociated with HIV-1 infection. *Cell Host Microbe* **14**:329-339.

Lozupone CA, Stombaugh J, Gonzalez A, Ackermann G, Wendel D, Vazquez-Baeza Y, Jansson JK, et al. 2013. Meta-analyses of studies of the human microbiota. *Genome Res* **23**:1704-1714.

Lund-Tønnesen S, Berstad A, Schreiner A, Midvedt T. 1998. *Clostridium difficile*-associated diarrhea treated with homologous feces. *Tidsskr Nor Laegeforen* **118**:1027-1030.

Lupp C, Roberston ML, Wickham ME, Sekirov I, Champion OL, Gaynor EC, Finlay BB. 2007. Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of *Enterobacteriaceae*. *Cell Host Microbe* **2**:119-129.

Lyras D, O'Connor JR, Howarth PM, Sambol SP, Carter GP, Phumoonna T, Poon R, et al. 2009. Toxin B is essential for virulence of *Clostridium difficile*. *Nature* **458**:1176-1179.

MacConnachie AA, Fox R, Kennedy DR, Seaton RA. 2009. Faecal transplant for recurrent *Clostridium difficile*-associated diarrhea: a UK case series. *QJM* **102**:781-784.

Magill SS, Edwards JR, Bamberg W, Beldavs ZG, Dumyati G, Kainer MA, Lynfield R, et al. 2014. Multistate point-prevalence survey of health care-associated infections. *N Engl J Med* **370**:1198-1208.

Mahony DE, Meier CE, Macdonald IA, Holdeman LV. 1977. Bile salt degradation by nonfermentative clostridia. *Appl Environ Microbiol* **34**:419-423.

Markelov A, Livert D, Kohli H. 2011. Predictors of fatal outcome after colectomy for fulminant *Clostridium difficile* colitis: a 10-year experience. *Am Surg* **77**:977-980.

Marsh JW, Arora R, Schlackman JL, Shutt KA, Curry SR, Harrison LH. 2012. Association of relapse of *Clostridium difficile* disease with BI/NAP1/027. *J Clin Microbiol* **50**:4078-4082.

McDonald D, Price MN, Goodrich J, Nawrocki EP, DeSantis TZ, Probst A, Andersen GL, et al. 2012. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME J* **6**:610-618.

McDonald D, Clemente JC, Kuczynski J, Rideout JR, Stombaugh J, Wendel D, Wilke A, et al. 2012. The Biological Observation Matrix (BIOM) format or: how I learned to stop worrying and love the ome-ome. *GigaScience* **1**:7.

McDonald LC, Killgore GE, Thompson A, Owens RC, Kazakova SV, Sambol SP, Johnson S, Gerding DN. 2005. An epidemic, toxin gene-variant strain of *Clostridium difficile*. *New Engl J Med* **353**:2433-2441.

Mekhjian HS, Phillips SF, Hofmann AF. 1979. Colonic absorption of unconjugated bile acids: perfusion studies in man. *Dig Dis Sci* **24**:545-550.

Merrigan M, Venugopal A, Mallozzi M, Roxas B, Viswanathan VK, Johnson S, Gerding DN, Vedantam G. 2010. Human hypervirulent *Clostridium difficile* strains exhibit increased sporulation as well as robust toxin production. *J Bateriol* **192**:490-4911.

Miller BA, Chen LF, Sexton DJ, Anderson DJ. 2011. Comparison on the burdens of hospital-onset, healthcare facility-associated *Clostridium difficile* infection due to methicillin-resistant *Staphylococcus aureus* in community hospitals. *Infect Control Hosp Epidemiol* **32**:387-390.

Moir A, Smith DA. 1990. The genetics of bacterial spore germination. *Annu Rev Microbiol* **44**:531-553.

Mulherin DW, Hutchison AM, Thomas GJ, Hansen RA, Childress DT. 2014. Concordance of the SHEA-IDSA severity classification for *Clostridium difficile* infection and the ATLAS bedside scoring system in hospitalized adult patients. *Infection* **42**:999-1005.

Nam HJ, Kang JK, Kim SK, Ahn KJ, Seok H, Park SJ, Chang JS, et al. 2010. *Clostridium difficile* toxin A decreases acetylation of tubulin, leading to microtubule depolymerization through activation of histone deacetylase 6, and this mediates acute inflammation. *J Biol Chem* **285**:32888-32896.

Nava GM, Stappenbeck TS. 2011. Diversity of the autochthonous colonic microbiota. *Gut Microbes* **2**:99-104.

Ng KM, Ferreyra JA, Higginbottom SK, Lynch JB, Kashyap PC, Gopinth S, Naidu N, et al. 2013. Microbiota-liberated host sugars facilitate post-antibiotic expansion of enteric pathogens. *Nature* **502**:96-99.

van Nood E, Vrieze A, Nieuwdorp M, Fuentes S, Zoetendal EG, de Vos WM, Visser CE, et al. 2013. Duodenal infusion of donor feces for recurrent *Clostridium difficile*. *N Engl J Med* **368**:407-415.

Padmanabhan P, Grosse J, Bakar Md Ali Asad A, Radda GK, Golay X. 2013. Gastrointestinal transit measurements in mice with ^{99m}Tc-DTPA-labeled activated charcoal using NanoSPECT-CT. *EJNMMI Research* **3**:60.

Paredes-Sabja D, Shen A, Sorg JA. 2014. *Clostridium difficile* spore biology: sporulation, germination, and spore structural proteins. *Trends Microbiol* **22**:406-416.

Patel LN, Schairer J, Shen B. 2014. Fecal transplantation therapy for *Clostridium difficile*-associated pouchitis. *Int J Colorectal Dis* **29**:263-264.

Pepin J, Alary ME, Valiquette L. 2005. Increasing risk of relapse after treatment of *Clostridium difficile* colitis in Quebec, Canada. *Clin Infect Dis* **40**:1591-1597.

Pereira FC, Saujet L, Tomé AR, Serrano M, Monot M, Couture-Tosi E, Martin-Verstraete I, et al. 2013. The spore differentiation pathway in the enteric pathogen *Clostridium difficile*. *PLoS Genet* **9**:e1003782.

Perelle S, Gibert M, Bourlioux P, Corthier G, Popoff MR. 1997. Production of a complete binary toxin (actin-specific ADP-ribosyltransferase) by *Clostridium difficile* CD196. *Infect Immun* **65**:1402-1407.

Petrof EO, Gloor GB, Vanner SJ, Weese SJ, Carter D, Daigneault MC, Brown EM, et al. 2013. Stool substitution transplant therapy for the eradication of *Clostridium difficile* infection: 'RePOOPulating' the gut. *Microbiome* **1**:3.

Poupon R. 2010. Primary biliary cirrhosis: a 2010 update. *J Hepatol* **52**:745-758.

Pryde SE, Duncan SH, Hold GL, Stewart CS, Flint HJ. 2002. The microbiology of butyrate formation in the human colon. *FEMS Microbiol Lett* **217**:133-139.

Reeves AE, Koenigsknecht MJ, Bergin IL, Young VB. 2012. Suppression of *Clostridiun difficile* in the gastrointestinal tracts of germfree mice inoculated with a murine isolate from the family *Lachnospiraceae*. *Infect Immun* **80**:3786-3794.

Ridaura VK, Faith JJ, Rey FE, Cheng J, Duncan AE, Kau AL, Griffin NW, et al. 2013. Gut microbiota from twins discordant for obesity modulate metabolism in mice. *Science* **341**:1241214.

Ridlon JM, Kang DJ, Hylemon PB. 2006. Bile salt biotransformations by human intestinal bacteria. *J Lipid Res* **47**:241-259.

Ridlon JM, Kang DJ, Hylemon PB. 2010. Isolation and characterization of a bile acid inducible 7α-dehydroxylating operon in *Clostridium hylemonae* TN271. *Anaerobe* **16**:137-146.

Rodrigues CM, Kren TB, Steer CJ, Setchell KD. 1995. The site-specific delivery of ursodeoxycholic acid to the rat colon by sulfate conjugation. *Gastroenterology* **109**:1835-1844.

Rossen NG, Fuentes S, van der Spek MJ, Tijssen J, Hartman JH, Duflou A, Löwenberg M, et al. 2015. Findings from a randomized controlled trial of fecal transplantation for patients with ulcerative colitis. *Gastroenterology* Mar 30. doi: 10.1053/j.gastro.2015.03.045.

Rupnik M, Avesani V, Janc M, von Eichel-Streiber C, Delmee M. 1998. A novel toxinotyping scheme and correlation of toxinotypes with serogroups of *Clostridium difficile* isolates. *J Clin Microbiol* **36**:2240-2247.

Russell DW, Setchell KD. 1992. Bile acid biosynthesis. *Biochemistry* **31**:4737-4749.

Saujet L, Pereira FC, Serrano M, Soutourina O, Monot M, Shelyakin PV, Gelfand MS, et al. 2013. Genome-wide analysis of cell type-specific gene transcription during spore formation in *Clostridium difficile*. *PLoS Genet* **9**:e1003756.

Schwan A, Sjolin S, Trottestam U. 1983. Relapsing *Clostridium difficile* enterocolitis cured by rectal infusion of homologous feces. *Lancet* **2**:845.

Sebaihia M, Wren BW, Mullany P, Fairweather NF, Minton N, Stabler R, Thomson NR, et al. 2006. The multidrug-resistant human pathogen *Clostridium difficile* has a highly mobile, mosaic genome. *Nat Genet* **38**:779-786.

Sebaihia M, Peck MW, Minton NP, Thomson NR, Holden MT, Mitchell WJ, Carter AT, et al. 2007. Genome sequence of a proteolytic (Group I) *Clostridium botulinum* strain Hall A and comparative analysis of the clostridial genomes. *Genome Res* **17**:1082-1092.

See I, Mu Y, Cohen J, Beldavs ZG, Winston LG, Dumyati G, Holzbauer S, et al. 2014. NAP1 strain type predicts outcomes from *Clostridium difficile* infection. *Clin Infect Dis* **58**:1394-1400.

Seekatz AM, Aas J, Gessert CE, Rubin TA, Saman DM, Bakken JS, Young VB. 2014. Recovery of the gut microbiome following fecal microbiota transplantation. *MBio* **5**:e00893-14.

Seril DN, Shen B. 2014. *Clostridium difficile* infection in patients with ileal pouches. *Am J Gastroenterol* **109**:941-947.

Setchell KDR. 1996. Sulfate conjugates of ursodeoxycholic acid, and their beneficial use in inflammatory disorders and other applications. US Patent CA2238040C, published 12 Jul 2004.

Setlow P. 2003. Spore germination. *Curr Opin Microbiol* **6**:550-556.

Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, et al. 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* **75**:7537-7541.

Shahinas D, Silverman M, Sittler T, Chiu C, Kim P, Allen-Vercoe E, Weese S, et al. 2012. Toward an understanding of changes in diversity associated with fecal microbiome transplantation based on 16S rRNA gene deep sequencing. *MBio* **3**:e00338-12.

Shankar V, Hamilton MJ, Khoruts A, Kilburn A, Unno T, Paliy O, Sadowsky MJ. 2014. Species and genus level resolution analysis of gut microbiota in *Clostridium difficile* patients following fecal microbiota transplantation. *Microbiome* **2**:13.

Shin A, Camilleri M, Vijayvargiya P, Busciglio I, Burton D, Ryks M, Rhoten D, et al. 2013. Bowel functions, fecal unconjugated primary and secondary bile acids, and colonic transit in patients with irritable bowel syndrome. *Clin Gastroenterol Hepatol* **11**:1270-1275.

Song Y, Garg S, Girotra M, Maddox C, von Rosenvinge EC, Dutta A, Dutta S, Frick WF. 2013. Microbiota dynamics in patients treated with fecal microbiota transplantation for recurrent *Clostridium difficile* infection. *PLoS ONE* **8**:e81330.

Sorg JA, Sonenshein AL. 2008. Bile salts and glycine as cogerminants for *Clostridium difficile* spores. *J Bacteriol* **190**:2505-2512.

Sorg JA, Sonenshein AL. 2009. Chenodeoxycholate is an inhibitor of *Clostridium difficile* spore germination. *J Bacteriol* **191**:1115-1117.

Sorg JA, Sonenshein AL. 2010. Inhibiting the initiation of *Clostridium difficile* spore germination using analogs of chenodeoxycholic acid, a bile acid. *J Bacteriol* **192**:4983-4990.

Spigaglia P, Mastrantonio P. 2002. Molecular analysis of the pathogenicity locus and polymorphism in the putative negative regulator of toxin production (TcdC) among *Clostridium difficile* clinical isolates. *J Clin Microbiol* **40**:3470-3475.

Steiner E, Dago AE, Young DI, Heap JT, Minton NP, Hoch JA, Young M. 2011. Multiple orphan histidine kinases interact directly with Spo0A to control the initiation of endospore formation in *Clostridium acetobutylicum*. *Mol Microbiol* **80**:641-654.

Stellwag EJ, Hylemon PB. 1978. Characterization of 7α-dehydroxylase in *Clostridium leptum*. *Am J Clin Nutr* **31**:S243-S247.

Stollman N, Smith M, Giovanelli A, Mendolia G, Burns L, Didyk E, Burgess J, et al. 2015. Frozen encapsulated stool in recurrent *Clostridium difficile*: exploring the role of pills in the treatment hierarchy of fecal microbiota transplant nonresponders. *Am J Gastroenterol* **110**:600-601.

Stubbs S, Rupnik M, Gibert M, Brazier J, Duerden B, Popoff M. 2000. Production of actin-specific ADP-ribosyltransferase (binary toxin) by strains of *Clostridium difficile*. *FEMS Microbiol Lett* **186**:307-312.

Surawicz CM, Alexander J. 2011. Treatment of refractory and recurrent *Clostridium difficile* infection. *Nat Rev Gastrenterol Hepatol* **8**:330-339.

Surawicz CM, Brandt LJ, Binion DG, Ananthakrishnan AN, Curry SR, Gilligan PH, McFarland LV, et al. 2013. Guidelines for diagnosis, treatment, and prevention of *Clostridium difficile* infections. *Am J Gastroenterol* **108**:478-498.

Swidsinski A, Weber J, Loening-Baucke V, Hale LP, Lochs H. 2005. Spatial organization and composition of the mucosal flora in patients with inflammatory bowel disease. *J Clin Microbiol* **43**:3380-3389.

Takamine F, Imamura T. 1995. Isolation and characterization of bile acid 7-dehydroxylating bacteria from human feces. *Microbiol Immunol* **39**:11-18.

Tamboli CP, Neut C, Desreumaux P, Colombel JF. 2004. Dysbiosis as a prerequisite for IBD. *Gut* **53**:1057.

Tannock GW, Munro K, Taylor C, Lawley B, Young W, Byrne B, Emery J, Louie T. 2010. A new macrocyclic antibiotic, fidaxomicin (OPT-80), causes less alteration to the bowel microbiota of *Clostridium difficile*-infected patients than does vancomycin. *Microbiology* **156**:3354-3359.

Theriot CM, Koumpouras CC, Carlson PE, Bergin II, Aronoff DM, Young VB. 2011. Cefoperazone-treated mice as an experimental platform to assess differential virulence of *Clostridium difficile* strains. *Gut Microbes* **2**:326-334.

Theriot CM, Koenigsknecht J, Carlson, Jr., PE, Hatton GE, Nelson AM, Li B, Huffnagle GB, Young VB. 2014. Antibiotic-induced shifts in the mouse gut microbiome and metabolome increase susceptibility to *Clostridium difficile* infection. *Nat Commun* **5**:3114.

Toda T, Ohi K, Kudo T, Yoshida T, Ikarashi N, Ito K, Sugiyama K. 2009. Antibiotics suppress Cyp3a in the mouse liver by reducing lithocholic acid-producing intestinal flora. *Yakagaku Zasshi* **129**:601-608.

Trubiano JA, Gardiner B, Kwong JC, Ward P, Testro AG, Charles PG. 2013. Faecal microbiota transplantation for severe *Clostridium difficile* infection in the intensive care unit. *Eur J Gastroenterol Hepatol* **25**:255-257.

Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* **444**:1027-1031.

Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. 2007. The human microbiome project. *Nature* **449**:804-810.

Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, Sogin ML, et al. 2009. A core gut microbiome in obese and lean twins. *Nature* **457**:480-484.

Tvede M, Rask-Madsen J. 1989. Bacteriotherapy for chronic relapsing *Clostridium difficile* diarrhea in six patients. *Lancet* **1**:1156-1160.

Underwood S, Guan S, Vijayasubhash V, Baines SD, Graham L, Lewis RJ, Wilcox MH, et al. 2009. Characterization of the sporulation initiation pathway of *Clostridium difficile* and its role in toxin production. *J Bacteriol* **191**:7296-7305.

Vardakas KZ, Polyzos KA, Patouni K, Rafailidis PI, Samonis G, Falagas ME. 2012. Treatment failure and recurrence of *Clostridium difficile* infection following

treatment with vancomycin or metronidazole: a systematic review of the evidence. *Int J Antimicrob Agents* **40**:1-8.

Vazquez-Baeza Y, Pirrung M, Gonzalez A, Knight R. 2013. EMPeror: a tool for visualizing high-throughput microbial community data. *GigaScience* **2**:16.

Voth DE, Ballard JD. 2005. *Clostridium difficile* toxins: mechanism of action and role in disease. *Clin Microbiol Rev* **18**:247-263.

Vrieze A, van Nood E, Holleman F, Salojärvi J, Kootte RS, Bartelsman JF, Dallinga-Thie GM, et al. 2012. Transfer of intestinal microbiota from lean donors increases insulin sensitivity in individuals with metabolic syndrome. *Gastroenterology* **143**:913-916.

Walker AS, Eyre DW, Wyllie DH, Dingle KE, Griffiths D, Shine B, Oakley S, et al. 2013. Relationship between bacterial strain type, host biomarkers, and mortality in *Clostridium difficile* infection. *Clin Infect Dis* **56**:1589-1600.

Wang T, Cai G, Qiu Y, Fei N, Zhang M, Pang X, Jia W, Cai S, Zhao L. 2012. Structural segregation of gut microbiota between colorectal cancer patients and healthy volunteers. *ISME J* **6**:320-329.

Warny M, Pepin J, Fang A, Killgore G, Thompson A, Brazier J, Frost E, McDonald LC. 2005. Toxin production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe. *Lancet* **366**:1079-1084.

Weingarden AR, Chen C, Bobr A, Yao D, Lu Y, Nelson VM, Sadowsky MJ, Khoruts A. 2014. Microbiota transplantation restores normal fecal bile acid composition in recurrent *Clostridium difficile* infection. *Am J Physiol Gastrointest Liver Physiol* **306**:G310-G319.

Wells JE, Berr F, Thomas LA, Dowling RH, Hylemon PB. 2000. Isolation and characterization of cholic acid 7α-dehydroxylating fecal bacteria from cholesterol gallstone patients. *J Hepatol* **32**:4-10.

Wells JE, Williams KB, Whitehead TR, Heuman DM, Hylemon PB. 2003. Development and application of a polymerase chain reaction assay for the detection and enumeration of bile acid 7α-dehydroxylating bacteria in human feces. *Clin Chim Acta* **331**:127-134.

Williams BL, Hornig M, Buie T, Bauman ML, Cho Paik M, Wick I, Bennett A, et al. 2011. Impaired carbohydrate digestion and transport and mucosal dysbiosis in the intestines of children with autism and gastrointestinal disturbances. *PLoS ONE* **6**:e24585.

Wilson KH, Kennedy MJ, Fekety FR. 1982. Use of sodium taurocholate to enhance spore recovery on a medium selective for *Clostridium difficile*. *J Clin Microbiol* **15**:443-446.

Wilson KH. 1983. Efficiency of various bile salt preparations for stimulation of *Clostridium difficile* spore germination. *J Clin Microbiol* **18**:1017-1019.

Wysowski DK. 2006. Increase in deaths related to enterocolitis due to *Clostridium difficile* in the United States, 1999-2002. *Public Health Rep* **121**:361-362.

Yasuda K, Oh K, Ren B, Tickle TL, Franzosa EA, Wachtman LM, Miller AD, et al. 2015. Biogeography of the intestinal mucosal and lumenal microbiome in the rhesus macaque. *Cell Host Microbe* **17**:385-391.

Yoon SS, Brandt LJ. 2010. Treatment of refractory/recurrent *C. difficile*-associated disease by donated stool transplanted via colonoscopy: a case series of 12 patients. *J Clin Gastroenterol* **44**:562-566.

You DM, Franzos MA, Holman RP. 2008. Successful treatment of fulminant *Clostridium difficile* infection with fecal bacteriotherapy. *Ann Intern Med* **148**:632-633.

Young VB, Raffals LH, Huse SM, Vital M, Dai D, Schloss PD, Brulc JM, et al. 2013. Multiphasic analysis of the temporal development of the distal gut microbiota in patients following ileal pouch anal anastomosis. *Microbiome* **1**:9.

Youngster I, Sauk J, Pindar C, Wilson RG, Kaplan JL, Smith MB, Alm EJ, et al. 2014. Fecal microbiota transplant for relapsing *Clostridium difficile* infection using a frozen inoculum from unrelated donors: a randomized, open-label, controlled pilot study. *Clin Infect Dis* **58**:1515-1522.

Youngster I, Russell GH, Pindar C, Ziv-Baran T, Sauk J, Hohmann EL. 2015. Oral, capsulized, frozen fecal microbiota transplantation for relapsing *Clostridium difficile* infection. *JAMA* **312**:1772-1778.

Zella GC, Hait EJ, Glavan T, Gevers D, Ward DV, Kitts CL, Korzenik JR. 2011. Distinct microbiome in pouchitis compared to healthy pouches in ulcerative colitis and familial adenomatous polyposis. *Inflamm Bowel Dis* **17**:1092-1100.

Zilberberg MD, Shorr AF, Kollef MH. 2008. Growth and geographic variation in hospitalizations with resistant infections. *Emerg Infect Dis* **14**:1756-1758.

Zoetendal EG, Rajilic-Stojanovic M, de Vos WM. 2008. High-throughput diversity and functionality analysis of the gastrointestinal tract microbiota. *Gut* **57**:1605-1615.

Zoetendal EG, Raes J, van den Bogert B, Arumugam M, Booijink CC, Troost FJ, Bork P, et al. 2012. The human small intestinal microbiota is driven by rapid uptake and conversion of simple carbohydrates. *ISME J* **6**:1415-1426.